

An investigation of the stem cell potential of skeletal muscle satellite cells

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Abstract

Satellite cells are defined by their position beneath the basal lamina of myofibres, and are a source of new myonuclei in adult skeletal muscles. However, other phenotypes also contribute to muscle regeneration, and the relative importance of satellite cells is not known. This work aimed to analyse the stem cell potential of satellite cells by formally investigating their contribution to muscle regeneration.

Myofibres isolated from extensor digitorum longus, soleus, and tibialis anterior muscles were found to have respective means of 7, 22 and 10 associated satellite cells. When a single myofibre was grafted into an irradiated dystrophic mouse muscle, the associated satellite cells underwent extensive, stem cell-like proliferation, generating progeny which sometimes gave rise to a cluster of more than 100 new myofibres. Cluster size varied according to the muscle group from which the graft was derived, but was not proportional to satellite cell number. Primary myoblasts derived from equivalent muscle groups did not undergo such extensive proliferation, or show inter-muscle variability, suggesting that stem cell activity is critically dependent on a component of the satellite cell niche. Single myofibres isolated from irradiated muscles were non-myogenic after grafting.

Satellite cells associated with single myofibres were found to generate new satellite cells in engrafted muscles, demonstrating that satellite cell compartment is maintained by self-renewal.

When single myofibre-engrafted muscles were damaged with myotoxin, graft-derived cells underwent rapid clonal expansion to regenerate compact clusters of donor-derived myofibres. The percentage of engrafted muscles containing identifiable donor-derived nuclei was increased after damage, showing that previously inactive cells had been recruited into an active myogenic program. Without experimentally-induced damage, frequency of muscle formation and cluster size were spontaneously augmented over time.

These findings demonstrate that satellite cells have several stem cell-like qualities, and thus constitute a self-sufficient and sustainable source of regeneration in adult muscles.

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List of Abbreviations

β-gal β-galactosidase

BrdU 5-bromo-2'-deoxyuridine

BMD Becker's muscular dystrophy

BSA bovine serum albumin

C57 Bl/10 C57 Bl/10ScSn strain wild type mouse

CEE chick embryo extract

CNS central nervous system

DAPI 4',6-diamidino-2-phenylindole fluorescent nuclear counterstain

DGC dystrophin-glycoprotein complex

DMD Duchenne's muscular dystrophy

DMEM Dulbecco's modified Eagle's medium with 4500 mg/l glucose

dy/dy C57Bl/6 J-dy2J mouse

EDL extensor digitorum longus muscle

EM electron microscopy

FDB flexor digitorum brevis muscle

FGFR4 fibroblast growth factor receptor 4

FGF fibroblast growth factor

GFP green fluorescent protein

GRMD Golden retriever muscular dystrophic dog

Gy Gray

HBSS Hank's balanced salts solution without calcium and magnesium

H & E haematoxylin and eosin

HFMD Hypertrophic feline muscular dystrophy

HGF hepatocyte growth factor

HI-FCS heat-inactivated foetal calf serum

HS horse serum

HSC haematopoietic stem cell(s)

IFN- γ interferon- γ

Ig immunoglobulin

LAM-PCR linear amplification-mediated PCR

L-glu L-glutamine

LGMD limb-girdle muscular dystrophy

Lin lineage antigens

LTR long-terminal repeat

mATPase myofibrillar adenosine triphosphatase

mdx C57 Bl/10ScSn-*mdx* dystrophic mouse

mdx-nude C57 Bl/10ScSn-*mdx* mouse bred onto a nude background

MPC muscle precursor cell(s)

MNF myocyte nuclear factor

MP main population

MRF myogenic regulatory factor

MTT myoblast transfer therapy

MyHC myosin heavy chain

NTR non-translated region

PAF paraformaldehyde

PCNA proliferating cell nuclear antigen

PBS phosphate buffered saline

pen/strep penicillin and streptomycin solution

P passage number

RT room temperature

Sca-1 stem cell antigen-1

SCID severe combined immune deficient

SCF stem cell factor

SD standard deviation of the mean

SE standard error

SP side population

TA tibialis anterior muscle

X-gal 5-bromo-4-chloro-3-indolyl-B-D-galactopyronoside

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Chapter 1

Introduction

1.1 General introduction

Adult skeletal muscle has a remarkable ability to regenerate functional tissue following damage (Studitsky 1964, Carlson 1968, 1970, Heslop *et al.* 2000, Luz *et al.* 2002). The progressive clinical decline of patients suffering from myopathies such as Duchenne's muscular dystrophy (DMD) is in part attributable to failure of the regenerative process (Dubowitz 1985, Dubowitz 1992, Rafael *et al.* 1997, Decary *et al.* 2000).

Satellite cells are anatomically defined by their position beneath the basal lamina of myofibres (Mauro 1961), and are thought to be the main source of new myonuclei in regenerating skeletal muscles (Moss & Leblond 1971, Snow 1978, Lipton & Schultz 1979, Rosenblatt *et al.* 1995, reviewed, Partridge 2002a). In addition, differentiated muscle can be derived from sources outside the satellite cell compartment, both from separate precursor populations thought to be resident within the tissue (Asakura *et al.* 2002, Polesskaya *et al.* 2003), and from circulatory phenotypes (Ferrari *et al.* 1998, Camargo *et al.* 2003). Circulating bone marrow-derived precursors have also been shown to generate anatomically-defined satellite cells (LaBarge & Blau 2002, Dreyfus *et al.* 2004). Although the satellite cell pool is maintained throughout adult life, the mechanism by which functional satellite cells are renewed is not fully understood (reviewed, Zammit & Beauchamp 2002). The findings of *in vitro* studies have shown that cultured muscle precursor cells (MPC) are capable of maintaining a reserve population during differentiation, predicting that satellite cells are capable of self-renewal (Baroffio *et al.* 1996, Yoshida *et al.* 1998, Zammit *et al.* 2004a). Similarly, transplants of MPC into mouse muscles both contribute to differentiated myofibres, and generate a reserve population of undifferentiated reserve cells (Yao and Kurachi 1993, Blaveri *et al.* 1999, Gross and Morgan 1999, Heslop *et al.* 2001), at least a proportion of which reside within the satellite cell compartment (Blaveri *et al.* 1999,

Heslop *et al.* 2001). However, the contribution of such cells to subsequent myotoxin-induced regeneration is modest in proportion to the original number of grafted cells (Gross & Morgan 1999). The poor ability of bone marrow grafts to contribute to myotoxin-induced regeneration (Camargo *et al.* 2003, Corbel *et al.* 2003) similarly suggests that bone marrow-derived satellite cells have insignificant functional potential. Consequently, although the regenerative ability of adult skeletal muscle (Studitsky 1964, Carlson 1968, 1970, Heslop *et al.* 2000, Luz *et al.* 2002) is strongly resonant of stem cell function, robust myogenic stem cell activity has not yet been localised to any defined phenotype.

Dissecting the relative contributions of the various putative myogenic precursors is key to understanding the mechanism of adult skeletal muscle repair. The role of the satellite cell compartment remains incompletely defined. Satellite cells could be committed, intermediate progenitor cells whose differentiation is temporarily blocked, and which have a limited proliferative capacity but are renewed by a separate stem cell population. Alternatively, satellite cells might be self-renewing adult stem cells which are themselves the basal origin of new myonuclei in regenerating muscles (**Fig. 1.2**). This thesis has aimed to analyse the stem cell potential of satellite cells by formally investigating their contribution to skeletal muscle regeneration.

1.2 Structure and origin of skeletal muscle

1.21 The anatomy of adult skeletal muscle

Skeletal or striated muscle is a highly specialised contractile tissue that is responsible for movement of the skeleton. It consists predominantly of elongated bundles of myofibres ('fasciculi') that contract to generate directional force (Zammit & Beauchamp 2001). Individual fibres are separated by delicate supporting tissue called endomysium. The several fasciculi that comprise each muscle are surrounded by the perimysium, a loose collagenous sheath, and the whole muscle body is encased in a denser collagenous sheath called the epimysium. These connective tissues are continuous with those of the tendons and muscle attachments and supply muscle with nerves and blood vessels (Getty 1975, Burkitt *et al.* 1993). (**Fig. 1.1**)

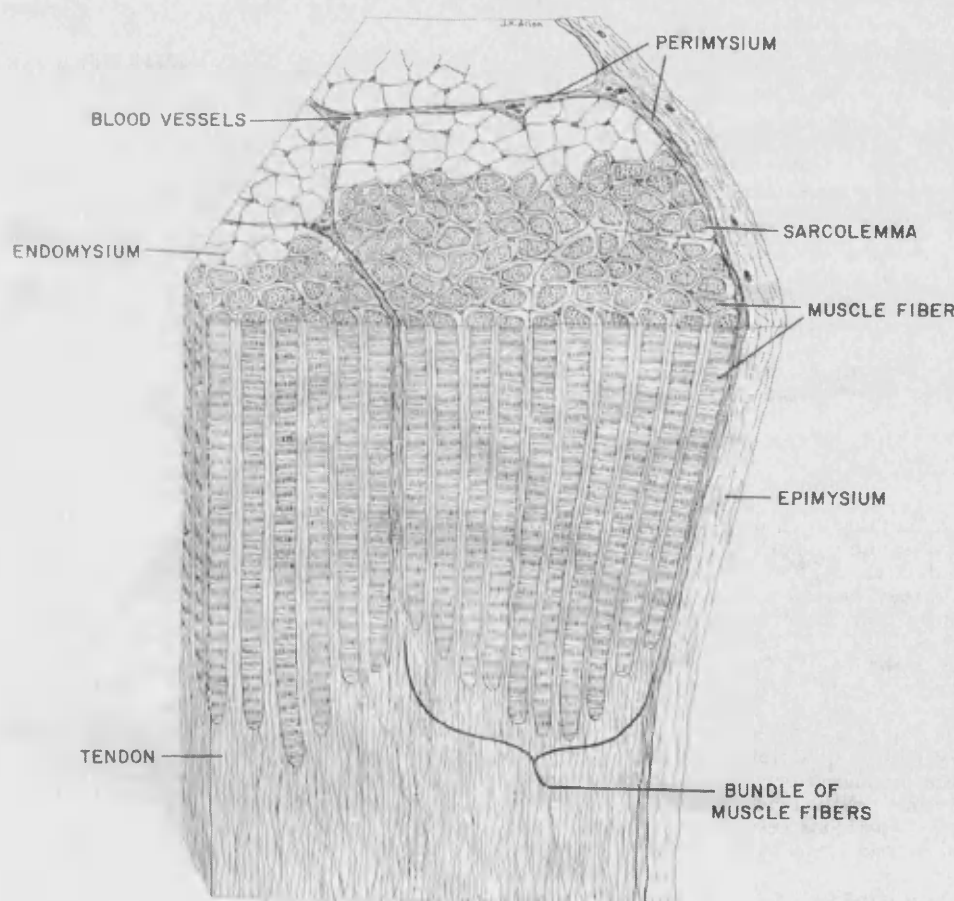


FIGURE 1-1. Organization of a skeletal muscle with its tendon.

Fig. 1.1. The anatomy of adult skeletal muscle. Reproduced from Getty (1975).

1.22 Myofibres as syncytia

Individual myofibres are highly differentiated cells that each consists of a contractile protein apparatus regulated by several hundred peripheral nuclei (Zammit & Beauchamp 2001). Counts of total nuclei in myofibres isolated from murine muscles have shown that on average, soleus myofibres have 458 nuclei (Zammit *et al.* 2002, unpublished data, J.D. Rosenblatt & T.A. Partridge), extensor digitorum longus (EDL) myofibres have 273 myonuclei (Zammit *et al.* 2002, unpublished data, J.D. Rosenblatt & T.A. Partridge), and tibialis anterior (TA) myofibres have around 600 nuclei (unpublished data, J.D. Rosenblatt & T.A. Partridge). In the rat, counts on serial histological sections have determined a mean of 105 nuclei per mm in soleus myofibres and 47 nuclei per mm in EDL myofibres (Schmalbruch & Lewis 2000). The much larger size of human muscles implies that their myofibres have even greater numbers of nuclei, although in contrast to rodents, many human myofibres are segmental and therefore each consist of several discrete cells (Bonavaud *et al.* 2002).

The post-mitotic nature of myonuclei was first demonstrated by Stockdale and Holtzer, who showed that ^3H -thymidine added to cultures of embryonic chick muscle cells was incorporated rapidly into the nuclei of mononucleate myoblasts, but only found in the nuclei of multinucleate myotubes after they had been generated by fusion of already-labelled mononucleate cells. Syncytial myofibres were not, therefore, formed by myonuclear replication in the absence of cytokinesis (Stockdale & Holtzer 1961). The original observation that muscle could be formed by differentiation of mononucleate cells probably comes from the early tissue culture experiments of Harrison, who reported that cross-striated fibres were generated from myoblasts emanating from tadpole myotome explants (Harrison 1910, cited in Lewis & Lewis 1917). These workers did not, however, observe these fibres to have been generated by fusion of several cells. Later, the *in vitro* formation of myotubes by fusion of mononucleate precursors was observed directly, in clonal cultures (Konigsburg 1961, 1963) by using time-lapse photography (Cooper & Konigsburg 1961), and by co-culturing of ^3H -thymidine labelled and unlabelled rat myoblasts (Yaffe & Feldman 1965). Evidence that these *in vitro* data recapitulated developmental myogenesis was derived from the generation of chimaeric mouse embryos from homozygotic parental strains expressing different isoforms of particular metabolic enzymes. The formation

of heteromeric isoforms of the enzymes in the chimaeras demonstrated that nuclei derived from both parental strains shared the common cytoplasm of mosaic myofibres (Mintz & Baker 1967, Gearhart & Mintz 1972). In adult tissue, treatment of rat muscle with ^3H -thymidine similarly showed that mononucleate precursors, but not myonuclei, incorporated radioactive label. However, when labelled cells were grafted into a new muscle, they became incorporated into host myofibres (Snow *et al.* 1978). Likewise, it was found that heteromeric enzymes were generated in engrafted adult mouse muscles when the donor and host strains expressed different homomeric enzyme isoforms (Partridge *et al.* 1978, reviewed, Partridge 1982). Together, these studies showed that throughout developmental myogenesis and adult regeneration, myofibres are derived from the fusion of mononucleate precursors and are themselves of a consistently postmitotic nature.

1.23 The developmental origin of skeletal muscle

(a) Developmental origin of skeletal muscles

The paraxial mesoderm of the vertebrate embryo gives rise to the somites, epithelial balls of cells that are located on either side of the neural tube and notochord, and are the origin of skeletal myogenesis (Chevallier *et al.* 1977). The ventro-medial region of each somite forms into the mesenchymal cells of the sclerotome, which later generates the axial skeleton and ribs. The dorso-lateral region of the somite gives rise to the dermomyotome from which skin and muscular tissues are derived. The epaxial (body wall) muscles are generated from the dorso-medial lip of the dermomyotome, whilst the hypaxial (limb, tongue and ventral wall) muscles derive from the dorso-lateral lip. Some craniofacial muscles originate from the unsegmented anterior paraxial mesoderm (reviewed, Christ & Ordahl 1995, Francis-West *et al.* 2003, Buckingham *et al.* 2003).

The dorso-lateral lip of the dermomyotome delaminates and gives rise to the limb muscle progenitors, in a process controlled by the paired box transcription factor Pax3 (Williams & Ordahl 1994, Daston *et al.* 1996). Pax3 is expressed throughout the somite during its formation, but becomes restricted to the dermomyotome as development progresses (Schubert *et al.* 2001). In Pax3^{-/-} *Spotch* mice (Auerbach 1954), cells do not delaminate from the hypaxial dermomyotome and the limb

muscles fail to develop (reviewed, Birchmeier & Brohmann 2000). Pax3 is required for expression of the hepatocyte growth factor (HGF) receptor, c-met (Epstein *et al.* 1996). Mice lacking either HGF or c-met similarly do not develop skeletal muscles (Schmidt *et al.* 1995, Bladt *et al.* 1995).

Muscle progenitors migrating into the limb bud express myogenic regulatory factors (MRFs), basic helix-loop-helix family transcription factors that define committed myogenic precursors (reviewed, Pownall *et al.* 2002). Of the four vertebrate MRFs (Myf5, MyoD, Mrf4 and myogenin), the *Myf5* gene is expressed earliest (Braun *et al.* 1992), followed by *MyoD* (reviewed, Pownall *et al.* 2002, Francis-West *et al.* 2003). *Myf5* is expressed in response to signalling from the neural tube, whilst *MyoD* expression is controlled by signals from the dorsal ectoderm (Cossu *et al.* 1996). Although relatively normal muscle development occurs in the absence of either *Myf5* (Tajbakhsh & Buckingham 1994) or *MyoD* (Rudnicki *et al.* 1993), in *Myf5*^{-/-}/*MyoD*^{-/-} double mutants the primitive progenitor population fails to form and skeletal muscles are absent (Rudnicki *et al.* 1993). Whilst either *Myf5* or *MyoD* are sufficient as determinants, they define different myogenic precursor cell subsets (Kablar *et al.* 2003) that adopt alternative non-muscle fates in mutants (Tajbakhsh *et al.* 1996b, Kablar *et al.* 2003). The MRF *myogenin* acts downstream of *Myf5* and *MyoD*, and is required to support muscle differentiation (Hasty *et al.* 1993, Rawls *et al.* 1995, reviewed, Francis-West *et al.* 2003). Mrf4 protein is also thought to be involved in differentiation (Naidu *et al.* 1995).

Migrating muscle precursors express the homeobox genes *Lbx1* and *Msx1*. *Lbx1* expression is initiated prior to delamination, maintained by migratory cells, and downregulated on differentiation. In *Lbx1* mutants, the migration of muscle precursors is inhibited and development of limb muscles severely restricted (Gross *et al.* 2000, reviewed, Birchmeier & Brohmann 2000). *Msx1* protein prevents premature differentiation and maintains proliferation (Houzelstein *et al.* 1999). Fibroblast growth factor receptor 4 (FGFR-4) is thought to be involved in the arrest of proliferation prior to differentiation (Marics *et al.* 2002).

Primary muscle fibres are formed in the first wave of embryonic myogenesis, in a nerve-independent process. After a second wave of proliferation, secondary foetal

muscle fibres are formed concomitantly with the initiation of innervation (Ontell & Kozeka 1984, Ross *et al.* 1987, Harris *et al.* 1989). Postnatal muscle is composed of myofibres of several distinct types, distinguishable by their expression of different isoforms of the contractile protein, myosin heavy chain (MyHC). Type I MyHC is expressed by slow-twitch oxidative myofibres, which in the mouse, are formed mainly in the primary wave of myogenesis. Type II MyHC isoforms are expressed by fast-twitch glycolytic myofibres, formed during the second wave (Condon *et al.* 1990, Narusawa *et al.* 1987, Harris *et al.* 1989). In birds, fast and slow MyHC genes are expressed by different lineages of committed embryonic myoblasts (DiMario *et al.* 1993). In mice, clonal analysis of muscle progenitor cells has shown that the embryonic origin of individual muscle groups is oligoclonal (Eloy-Trinquet & Nicolas 2002, reviewed, Buckingham *et al.* 2003).

(b) The developmental origin of satellite cells

A subset of undifferentiated muscle progenitors persists in adult muscle. First identified in the frog (Mauro 1961, Katz 1961), these cells are defined by their anatomical position between the basal lamina and sarcolemma of myofibres, and are termed satellite cells (Mauro 1961). In mouse embryos, satellite cells first appear 17.5 days post-coitum. Their developmental origin remains enigmatic (reviewed, Buckingham 2003). Clonal analysis of cells derived from chick embryos at various stages of development has suggested that in birds, satellite cells comprise a functionally-distinct lineage by the mid-foetal stage of development (Feldman & Stockdale 1992). Experimental grafting of quail somitic mesoderm tissue into the somitic mesoderm of chick embryos suggested that like differentiated muscle fibres, satellite cells are of somitic origin (Armand *et al.* 1983). However, more recent work has suggested that satellite cells may alternatively be derived from foetal mesoangioblasts originating in the dorsal aorta (De Angelis *et al.* 1999, Minasi *et al.* 2002, Sampaloesi *et al.* 2003). When quail or mouse embryonic aortas were transplanted into chick embryos, associated cells were found to contribute to a variety of mesodermal tissues including smooth, cardiac and skeletal muscle. Clonal progeny of transplanted cells were capable of undergoing extensive *in vitro* expansion without subsequent loss of multipotency on grafting into a second host (Minasi *et al.* 2002). The hypothesis that satellite cells may be derived from such vascular stem cells is supported by the finding that like foetal mesoangioblasts (De Angelis *et al.* 1999),

adult satellite cells express certain endothelial markers such as the haematopoietic differentiation antigen, CD34 (Beauchamp *et al.* 2000).

Pax3 expression is retained in some satellite cells, notably those of the gracilis muscle (reviewed, Buckingham *et al.* 2003). In addition, the majority of satellite cells express another closely related paired-box transcription factor, *Pax7* (Seale *et al.* 2000), which is also expressed by part of the dermomyotome during embryonic development (Mansouri *et al.* 1996). In *Pax7*^{-/-} mice there is no overt embryonic muscle defect (Mansouri *et al.* 1996) but the adult satellite cell compartment is substantially reduced (Seale *et al.* 2000). This was originally interpreted as evidence that *Pax7* is required for satellite cell specification (Seale *et al.* 2000). However, a recent study has shown that juvenile *Pax7*^{-/-} mice have relatively normal numbers of satellite cells, which do, however, decline as animals reach maturity. This implies that *Pax7* is not an absolute requirement for satellite cell specification, but is required for postnatal satellite cell renewal (Oustanina *et al.* 2004). Transfection of *Pax7* into previously uncommitted muscle-derived cells has been shown to be sufficient to induce myogenic commitment (Seale *et al.* 2004).

1.24 Expression of MyHC isoforms in developing and adult muscles

Fast and slow fibre types are distinct both in terms of their contractile speed and the pH stability of myofibrillar adenosine triphosphatase (mATPase) activity (Bàràny 1967, reviewed, Pette & Staron 2000, 2001). mATPase resides in the heavy chain portion of myosin molecules, and histochemically-detectable differences in mATPase activity directly correlate with specific MyHC isoform profiles. Myosin light chains and other proteins also exhibit fibre type specific expression profiles (reviewed, Pette & Staron 2000, 2001).

MyHC proteins are a major component of the cytoskeletal apparatus of myofibres. Eight MyHC protein isoforms have been identified in mammals, each of which is encoded by a different gene, which are regulated by different pathways (Allen *et al.* 2001). Embryonic myosin and neonatal (perinatal) myosin are predominantly expressed during development. Adult tissues express two isoforms of Type I (slow) MyHC, α and β . Type I α MyHC is expressed almost exclusively in cardiac muscle,

whilst Type I β MyHC is found in both cardiac muscle cells and slow-twitch oxidative muscle fibres such as those of the murine soleus. There are three adult isoforms of Type II (fast) MyHC, IIa, IIb and IIx (IIId). An additional isoform is specific to extraocular and pharyngeal muscles. The three adult Type II isoforms contribute the vast majority expression in murine skeletal muscles, with the IIb isoform alone forming >70% of total MyHC. Expression of IIa and IIx isoforms is also, however, high in certain muscles such as the soleus and diaphragm (reviewed, Pette & Staron 2000, 2001, Agbulut *et al.* 2003).

The earliest myofibres formed in the somitic myotome initially express embryonic myosin mRNA, shortly followed by expression of slow myosin mRNA, but not neonatal myosin mRNA (Sacks *et al.* 2003). During later embryonic and early postnatal development, expression of embryonic and neonatal MyHC isoforms predominates, and only trace levels of adult MyHC mRNAs are detectable (Lu *et al.* 1999). Although there is evidence that during development, myoblasts have an intrinsic commitment to particular patterns of myosin expression (Miller & Stockdale 1986), transplantation of clones of foetal myoblasts of known MyHC profile into chick limbs has demonstrated that the host environment can override this commitment and induce expression of a myosin profile characteristic of the new environment (Robson & Hughes 1999). In newborn muscle, the adult slow Type I β isoform makes a significant contribution to total MyHC protein, but adult fast MyHC isoforms are not co-expressed at high levels until about seven days after birth. By 21 days after birth, embryonic myosin is not expressed, but expression of neonatal myosin persists in some muscles such as the soleus and diaphragm, whilst it is absent in the EDL, gastrocnemius and plantaris. Neonatal myosin is virtually absent from adult muscles but persists in certain craniofacial muscles such as the masseter (Allen & Leinwand 2001, Agbulut *et al.* 2003).

Adult muscles contain both pure fibre types, which express a single MyHC isoform, and hybrid fibre types, which express two MyHC isoforms (reviewed, Pette & Staron 2000, Stephenson 2001). In hybrid fibres one isoform usually predominates. Hybrid fibre types include Type I/IIA (MyHC I β >MyHC IIa), Type IIA/I (MyHC IIa>MyHC I β), Type IIAD (MyHC IIa>MyHC IIId), Type IIDB (MyHC IIId>MyHC IIb) and Type IIBD (MyHC IIb>MyHC IIId) (reviewed, Pette &

Staron 2000). Type I β slow myosin and is rarely co-expressed with the fastest myosin isoform, Type IIb. However, co-expression of these isoforms has been observed in regenerated soleus muscle after transplantation into the EDL muscle bed (Snoj-Cvetko *et al.* 1996). Regulation of fibre type expression is primarily determined by the electrical activity of the associated nerve (Buller *et al.* 1960, reviewed, Hughes & Salinas 1999), though adult muscle fibres and myoblasts exhibit certain characteristics which are retained independently of their type of innervation (reviewed, Hughes & Salinas 1999).

1.3 Adult stem cells

1.31 Definition of a stem cell

A stem cell is functionally-defined as a cell which has the sustained ability to both self-renew and generate at least one differentiated cell type, and accordingly the potential to robustly reconstitute a given tissue *in vivo* (Lajtha 1979, Moore 1979, Morrison *et al.* 1997, Anderson *et al.* 2001). In comparison, a progenitor cell also has the ability to generate one or more differentiated cell types, but does not have the ability to self-renew, although it may undergo extensive proliferation prior to differentiation (Elwood 2004). Characteristic attributes of stem cells include rarity, the potential to generate several distinct differentiated phenotypes (multipotency), the ability to exist in a state of reversible mitotic quiescence, and the ability to undergo asymmetric division, thereby both replacing themselves and generating a differentiation-committed lineage (Hall & Watt 1989, Potten & Loeffler 1990, Morrison *et al.* 1997, Weissman *et al.* 2000 Marshman *et al.* 2002). The best-characterized adult stem cells are those of the continuously-renewing tissues of the haematopoietic system, skin and gut epithelium.

1.32 Stem cells of adult tissues

(a) Haematopoietic system

The cells of the mammalian lymphohaematopoietic system are defined by their pan-expression of leukocyte common antigen, a protein tyrosine phosphatase commonly known as CD45 (Sunderland *et al.* 1979, Dalchau & Fabre 1981, reviewed, Trowbridge & Thomas 1994). Differentiated haematopoietic cells have a finite lifespan, and in man, are renewed at an estimated rate of 10^{11} to 10^{12} cells per day

(reviewed, Elwood 2004). Haematopoietic stem cells (HSC) are defined by their clonogenic ability to generate all the lineages of the lymphohaematopoietic system, including B and T lymphocytes, erythrocytes, megakaryocytes, basophils, eosinophils, neutrophils and macrophages (Till & McCulloch 1961, reviewed, Lajtha 1979, Weissman 2000, Smith 2003, Szilvassy 2003). They constitute the canonical example of an adult stem cell. The self-renewal capacity of HSC is such that a single grafted cell can home to the spleen, proliferate, and repopulate and maintain the entire haematopoietic system of a lethally-irradiated mouse (Smith *et al.* 1991, Osawa *et al.* 1996, Matsuzaki *et al.* 2004). Staining of whole bone marrow with the vital dye Hoechst 33342 identifies a small subset of cells, termed side population (SP), which has very strong dye efflux activity and contains the vast majority of HSC (Goodell *et al.* 1996). The molecular determinant of the SP phenotype is thought to be expression of the ABC transporter Bcrp1 (Zhou *et al.* 2001, 2002). The majority of SP is quiescent (phase G₀ of the cell cycle), but at any given timepoint about 3% of cells are cycling (phase S-G₂M); all, however, have been shown to have repopulation potential in lethally-irradiated mice (Goodell *et al.* 1996). HSC can also be isolated according to their antigen expression profiles. About half of all SP are negative for expression of the glycoprotein differentiation antigen CD34, but positive for expression for stem cell antigen-1 (Sca-1) and the stem cell factor (SCF) transmembrane receptor c-kit. This is considered to be a primitive phenotype (Pearce *et al.* 2004). Murine HSC are characteristically negative for expression of a panel of lineage antigens (Lin) present on the differentiated cells of the haematopoietic system (reviewed, Szilvassy 2003). A recent study by Matsuzaki *et al.* (2004) described a population of murine bone marrow cells defined by their CD34⁻ c-Kit⁺ Sca-1⁺ Lin⁻ expression profile and very strong Hoechst dye efflux activity. Single cells derived from such populations were found to be capable of multilineage haematopoietic reconstitution in 96% of lethally-irradiated mice, suggesting that the most primitive HSC have near absolute repopulating efficiency. These data additionally imply that HSC have an extraordinarily good ability to home to the spleen, the environment required for their survival and proliferation (Matsuzaki *et al.* 2004).

Primitive HSC exist in a state of reversible mitotic quiescence, but are not truly dormant (Bradford *et al.* 1997, Cheshier *et al.* 1999). Whilst at least 75% of HSC are in G₀ at any one timepoint (Cheshier *et al.* 1999), they are recruited back into the cell

cycle at regular intervals. The majority of HSC cycle at least once during a thirty day period, as shown by both bromodeoxyuridine (BrdU) labelling of DNA precursors (Cheshier *et al.* 1999) and measurement of relative levels of rhodamine and Hoechst dye fluorescence (Bradford *et al.* 1997). The sustained self-renewal ability of HSC is linked to their production of the enzyme telomerase, which conserves the length of telomeres, the non-encoding regions of DNA capping the ends of chromosomes. During replication, telomeres position chromosomes within the nucleus and prevent DNA from degrading, fusing or rearranging. Telomeres are therefore essential to the maintenance of chromosomal stability and their shortening accordingly results in cell crisis and senescence. Whilst telomere length and telomerase expression are increased in both HSC and transformed cancerous cells, progenitor and differentiated cells have only minimal levels of telomerase activity (reviewed, Elwood 2004). It has been demonstrated that telomere length can be directly correlated with the known proliferative potential of different subpopulations of haematopoietic cells (Van Ziffle *et al.* 2003). HSC telomere length declines with age suggesting that replicative potential, though sustained, is finite (reviewed, Elwood 2004).

(b) Intestinal epithelium

The intestinal lumen is lined with simple columnar epithelium formed into finger-like villi that harbour the differentiated cells involved in nutrient absorption: enterocytes, mucus secreting goblet cells and enteroendocrine cells. Each villus is surrounded by invaginated crypts containing multipotential stem cells that continuously renew the differentiated phenotypes. Stem cells in the lower portion of each crypt generate progeny that form a coherent functional and anatomical hierarchy in which they migrate upwards out of the crypt, differentiate into the tissue of the villus and are eventually sloughed away through an apical extrusion zone. Some progeny migrate downwards into the base of the pit and become anti-microbial Paneth cells (reviewed, Marshman *et al.* 2002, Sancho *et al.* 2003, Potten 2004). Stem cells can be identified by their anatomical position in the crypt hierarchy (reviewed, Marshman *et al.* 2002), their retention of radioactive label (Potten *et al.* 1978, Potten *et al.* 2002), and their expression of the RNA binding protein musashi-1, which also marks primitive progenitor cells (Potten *et al.* 2004).

Intestinal epithelial stem cells divide approximately once a day, producing one daughter cell and one new stem cell. The daughter cell proliferates through about three population doublings forming a 'transit population' of progenitors that subsequently commits to differentiation and undergoes limited further proliferation (reviewed, Marshman *et al.* 2002, Sancho *et al.* 2003). If the stem cell population is ablated, the undifferentiated transit cells can reassume their full stem cell potential and clonogenically repopulate the whole crypt. Removal of even one stem cell triggers immediate cell-cycle perturbations that are likely to result in rapid restoration of stem cell number (reviewed, Marshman *et al.* 2002, Potten 2004). In contrast, ablation of the Paneth cell population has no effect on the proliferative status of stem cells (Stappenbeck *et al.* 2003). Studies using chimaeric mice have shown that although intestinal crypts are oligoclonal during the neonatal period, they become monoclonal within two weeks postpartum (Schmidt *et al.* 1988). Examination of DNA methylation tagging patterns has suggested that human colonic crypts contain multiple stem cells which periodically go through 'bottlenecks' whereby most stem cells become depleted, and the niche is subsequently reconstituted by the repeated self-renewal of a small number of survivors (Yatabe *et al.* 2001).

Mouse intestinal epithelial stem cells ultimately undergo approximately 1,000 population doublings, but show no loss of proliferative potential and rarely develop neoplastic mutations (reviewed, Marshman *et al.* 2002). Intestinal epithelial stem cells (Potten *et al.* 2002) and other asymmetrically-dividing stem cell-like lines (Merok *et al.* 2002) protect themselves from acquiring replication-induced errors by selective sorting of template and newly-synthesised DNA strands to ensure consistent retention of the original template strand by the renewed stem cell (Potten *et al.* 2002, Merok *et al.* 2002). Despite their sustained proliferative ability *in vivo*, isolated intestinal epithelial stem cells cannot be cultured for prolonged periods *in vitro*. They can, however, be successfully cultured within intact crypts, suggesting that the crypt microenvironment is essential to the maintenance of a functional stem cell phenotype (reviewed, Kaeffer 2002).

(c) Skin epidermis

The main cellular constituents of the adult skin epidermis are specialised epithelial cells called keratinocytes, which form a cohesive barrier and continuously renew the epidermal layer (reviewed, Gambardella & Barrendon 2002). Skin epithelial stem cells are located in the bulge area of hair follicles (Michel *et al.* 1996, Morris & Potten 1999, Blanpain *et al.* 2004) and in the interfollicular basal layer of the epidermis (Bickenbach 1981), where they can be identified by their long-term ability to retain labels such as ^3H -thymidine and BrdU (Bickenbach 1981, Morris & Potten 1999) and their expression of keratin 19 (Michel *et al.* 1996). Similarly to intestinal epithelial stem cells, skin epithelial stem cells both self-renew and give rise to daughter 'transient amplifying' progenitor cells that have a finite proliferative life before they exit the basal compartment and differentiate into mature keratinocytes (reviewed, Gambardella & Barrendon 2003). Skin epithelial stem cells have the ability to clonally regenerate all the epithelial cell types present in the hair follicle, as demonstrated both by gene targeting of putative stem cells with an inducible *Cre* recombinase construct (Morris *et al.* 2004) and transplantation of clonal cell populations (Blanpain *et al.* 2004). In addition, *in utero* transplantation experiments have shown that grafted epidermal stem cells (but not transient amplifying cells) can contribute to several different lineages during embryonic development (Liang & Bickenbach 2002).

Two distinct populations of multipotent skin stem cells have been identified within the bulge region of hair follicles: a basal population which is attached to the basal lamina of the extracellular matrix, and a suprabasal population which is detached and possibly derived from the attached population (Blanpain *et al.* 2004). The basal lamina appears to have a reversible proliferation-inhibitory effect such that basal cells undergo less divisions than suprabasal cells. This suggests that the basal lamina has a role in maintaining the niche in a quiescent state (Blanpain *et al.* 2004).

Epidermal cells contain an SP subset that excludes Hoechst dye (Terunuma *et al.* 2003, Triel *et al.* 2004). However, unlike blood SP, these cells do not exhibit stem cell characteristics (Triel *et al.* 2004) and also do not correspond to the label-retaining population (Terunuma *et al.* 2003, Triel *et al.* 2004).

(d) The nervous system

Neural stem cells are defined by their ability to both self-renew and give rise to the three main differentiated phenotypes found in the central nervous system (CNS): neurons, astrocytes and oligodendrocytes (reviewed, Temple & Alvarez-Buyella 1999, Kennea & Mehmet 2002). Although the adult CNS has a relatively poor ability to regenerate functional tissue after injury, it has been possible to isolate neural stem cells from two areas of the brain: the subventricular zone (Lois & Alvarez-Buylla 1993) and the subgranular zone of the hippocampus (Seaburg & van der Kooy 2002, Song *et al.* 2002a). Whilst proliferation competent cells are resident in other areas of the brain, they do not generate neurons (Horner *et al.* 2000, Kornack & Rakic 2001).

Clusters of dividing neural stem cells have been found to associate closely with the vasculature, suggesting that adult neurogenesis is likely to occur within an angiogenic environment (Palmer *et al.* 2000). A recent *in vitro* study has shown that endothelial cells (but not smooth muscle cells) of the vasculature release soluble factors which inhibit differentiation and stimulate self-renewal of neural stem cells (Shen *et al.* 2004). In addition, it has been demonstrated that differentiated astrocytes regulate neural stem cells in the adult hippocampus by stimulating them to undergo neuronal specification (Song *et al.* 2002b). These findings suggest that as in the intestinal (reviewed, Kaeffer 2002) and skin (Blanpain *et al.* 2004) epithelia, stem cell behaviour is critically determined by interaction with other, differentiated phenotypes.

1.33 Adult stem cell metaplasia

Adult urodele amphibians have a remarkable ability to regenerate complex anatomical structures, including whole limbs, jaws, heart and the lens and retinas of eyes. This process occurs through re-specification of differentiated cells into tissue-specific, replication-competent progenitor cells (reviewed, Brockes & Kumar 2002). The demonstration that a viable animal can be generated from the nucleus of a differentiated adult cell is unequivocal evidence that mammalian adult nuclei can also undergo developmental reprogramming, and, albeit under particular experimental conditions, even regain totipotency (Wilmut *et al.* 1997). However, it is not known whether embryonic lineage commitment is ever recapitulated in the absence of such

experimental manipulation, or whether all mammalian adult cells, including stem cells, are normally lineage restricted.

Metaplasia is defined as the process whereby one differentiated or precursor cell type is converted to another. Transdifferentiation is a form of metaplasia restricted to already-differentiated cells (reviewed, Tosh & Slack 2002). In the embryo, differentiated liver cells can be derived from transdifferentiation of differentiated pancreatic cells (Tosh *et al.* 2002, Shen *et al.* 2003). In the adult, the differentiated glomerular and tubular cells of the kidney are capable of reverting to a more primitive mesenchymal phenotype in response to injury, and subsequently of regenerating limited amounts of functional tissue (reviewed, Meguid El Nahas 2003). And in tissue culture, differentiated post-mitotic myotubes derived from immortalised mouse cells can also be induced to dedifferentiate and re-enter the cell cycle, either in response to forced over-expression of the homeobox gene *Msx1* (Odelburg *et al.* 2000), or after treatment with the purine-based chemical myoseverin (Rosania *et al.* 2000). When generated by forced *Msx1* over-expression, these re-derived progenitors have been reported to differentiate into cells of both the myogenic, and adipogenic and osteogenic lineages (Odelburg *et al.* 2000). Whilst there are a few other examples of adult metaplasia *in vivo*, notably within epithelial lineages (Slack 1985, 1986), recent studies have particularly focused on the unique ability of bone marrow-derived cells to contribute to a variety of non-haematopoietic tissues.

It has been demonstrated that intravenously grafted bone marrow cells can at low frequency contribute to skeletal muscle (Ferrari *et al.* 1998, Brazelton *et al.* 2003, Camargo *et al.* 2003), myocardium (Orlic *et al.* 2002, Alvarez-Dolado *et al.* 2003, Nygren *et al.* 2004), liver (Lagasse *et al.* 2000, Korbaling *et al.* 2002, Vassilopoulos *et al.* 2003, Wulf *et al.* 2003, Alvarez-Dolado *et al.* 2003, Camargo *et al.* 2004a), lung (Krause *et al.* 2001), skin (Krause *et al.* 2001, Korbaling *et al.* 2002, Kataoka *et al.* 2003), gastrointestinal tract (Krause *et al.* 2001, Korbaling *et al.* 2002), and in the brain, neuron-type cells (Brazelton *et al.* 2000, Mezey *et al.* 2000), Purkinje neurons (Weimann *et al.* 2003a, 2003b, Alvarez-Dolado *et al.* 2003) and microglial and perivascular cells (Hess *et al.* 2004). Whilst the use of whole bone marrow grafts in many of these studies made it difficult to identify the precise subset of cells involved, some workers report transplantation of a single, genetically-marked cell to show that

defined HSC can both clonally repopulate the haematopoietic system, and contribute to liver, lung, gastrointestinal tract and skin (Krause *et al.* 2001) or to skeletal muscle fibres (Camargo *et al.* 2003, Corbel *et al.* 2003). In all of the above studies, engraftment potential was maximised by whole body irradiation of recipients. In an alternative approach, circulatory cell metaplasia has also been investigated using mice surgically joined in parabiotic pairs. In the absence of whole body irradiation, it was found that despite substantial evidence of blood chimaerism, the contribution of HSC progeny to other tissues was almost non-existent (Wagers *et al.* 2002).

In tissue culture, bone marrow cells can adopt the phenotype of embryonic stem cells as a result of spontaneous nuclear fusion (Ying *et al.* 2002, Terada *et al.* 2002). Data from several studies have demonstrated that *in vivo*, many apparent metaplastic events are also attributable to fusion, whereby bone marrow cells form heterokaryons with Purkinje neurons (Weimann *et al.* 2003, Alvarez-Dolado *et al.* 2003), or are incorporated into already multinucleate differentiated phenotypes such as hepatocytes (Vassilopoulos *et al.* 2003, Alvarez-Dolado *et al.* 2003, Camargo *et al.* 2004a), skeletal muscle fibres (Camargo *et al.* 2003) and cardiomyocytes (Alvarez-Dolado *et al.* 2003, Nygren *et al.* 2004). In skeletal muscle (Camargo *et al.* 2003) and liver (Camargo *et al.* 2004a) lineage-tracking strategies have been used to show that cytoplasmic fusion occurs through intermediate precursor cells of the myeloid lineage, probably macrophages (Camargo *et al.* 2003, Camargo *et al.* 2004a, reviewed, Camargo *et al.* 2004b). Another study found that bone marrow-derived cells present in damaged myocardium failed to express markers of cardiac differentiation, and retained their haematopoietic lineage commitment. The number of cells present in injured hearts was found to decrease with time (Balsam *et al.* 2004). Taken together, these findings seem to imply that bone marrow cells do not undergo genuine metaplasia, and do not generate non-haematopoietic tissues via tissue-specific, replication-competent progenitor cells (reviewed, Vassilopoulos & Russell 2003, Camargo *et al.* 2004b).

The use of lineage non-specific markers (such as Y chromosome or other ubiquitously-expressed genetic markers) in a large proportion of studies has led to a paucity of data where stochastic cell fusion can be excluded as the mechanism by which cells contributed to other tissues (reviewed, Vassilopoulos & Russell 2003,

Camargo *et al.* 2004b). Nevertheless, it has been demonstrated that bone marrow cells can adopt the anatomical position (Gussoni *et al.* 1999, Fukada *et al.* 2002, LaBarge & Blau 2002, Dreyfus *et al.* 2004) and phenotypic expression profile (Dreyfus *et al.* 2004) of satellite cells, the committed precursors of differentiated muscle. And in addition, it has been shown that bone marrow cells can generate hepatic SP, which may have the ability to participate in liver regeneration (Wulf *et al.* 2003). Functional studies will be required to resolve these apparent anomalies.

1.4 Regeneration of adult skeletal muscle

1.41 Evidence for the regenerative ability of adult muscle

The most striking descriptions of adult skeletal muscle regeneration are derived from the early grafting experiments of Studitsky and Carlson (reviewed, Carlson 1986). Studitsky and his colleagues developed an experimental protocol whereby the muscle of a small animal was removed, finely minced to a “semi-liquid porridge” state, and then grafted back into the muscle bed. Remarkably, they found that within a few weeks, the minced tissue regenerated a model of the removed muscle consisting of bundles of innervated myofibres that were, moreover, capable of contraction in response to nervous stimuli. This phenomenon was demonstrated in the biceps brachii muscles of chicks and pigeons and the gastrocnemius muscles of rats and mice. In addition, it was shown that in some cases, not only autografts (grafts of tissue back into the same animal), but also homografts (grafts between siblings) were capable of re-forming functional tissue (Studitsky 1964). These findings were subsequently reproduced in similar experiments by Carlson, using frog (Carlson 1968) and rat (Carlson 1968, Carlson & Gutmann 1972) gastrocnemius autografts, and rat gastrocnemius homografts (Carlson 1970).

Studitsky's group also developed the technique of free muscle grafting (Bosova 1962, cited in Carlson 1986), in which a muscle is completely excised from its bed, and then either replaced, or sutured into a different site in the same host. The severing of associated tendons, nerves and blood vessels causes the muscle to undergo a reproducible series of degenerative and regenerative changes, during which myofibres become necrotic, are phagocytosed by macrophages, and are subsequently replaced by immature myotubes that develop into mature innervated myofibres. In the rat, this

cycle of degeneration and regeneration takes about two months, whereas in larger animals such as the cat, it takes about six months (Carlson *et al.* 1979, reviewed, Carlson 1986). In humans, immunohistochemical examination of biopsies derived from clinically transplanted muscles has demonstrated the presence of newly-formed myofibres several months post-operatively, implying that the process of regeneration also persists for an extended period in comparison to rodent muscles (Yoshimura *et al.* 1998b). There is, however, a lack of published data that describes the regenerative ability of normal human muscles, presumably because of the practical problems of conducting such studies.

Mouse skeletal muscles appear to have the ability to regenerate themselves repeatedly. After fifty weekly injections of the myotoxic drug bupivacaine, the TA muscles of wild-type mice were found to have maintained a total myofibre number equivalent to that in non-injured muscles (Luz *et al.* 2002).

Skeletal muscle maintains some regenerative ability even after irradiation. In the rat, it has been reported that whilst 1.5 Gray (Gy) of local irradiation disables the normal regenerative ability of muscle tissue, it is not sufficient to suppress the regenerative ability of minced muscle autografts (Studitsky 1988). In the mouse, local treatment of juvenile mouse muscles with 18 Gy irradiation results in a long-term growth and repair deficit (Wakeford *et al.* 1991, Quinlan *et al.* 1995). But when irradiated muscles are severely damaged by injection of the myotoxin notexin, they retain the ability to regenerate effectively (Heslop *et al.* 2000). If mouse muscles are locally treated with a larger dose of 25 Gy irradiation, this regenerative response is largely lost (Heslop *et al.* 2000). Taken together, these findings imply that adult rodent skeletal muscle employs at least two efficient regenerative mechanisms, one of which is sensitive to irradiation, and one of which is relatively resistant to irradiation but requires the stimulus of acute damage.

1.42 The role of satellite cells in muscle regeneration

(a) The contribution of satellite cells to skeletal muscle regeneration

Satellite cells are believed to be the main source of new myonuclei in regenerating muscles (Moss & Leblond 1971, Snow 1978, Lipton & Schultz 1979, reviewed, Partridge 2002a). A few hours after ^3H -thymidine was administered to growing rats, electron microscopy (EM) studies showed that label could be identified in satellite cell nuclei, but never myonuclei. The spontaneous appearance of labelled myonuclei at later timepoints demonstrated that the labelled satellite cells had subsequently contributed to myofibres (Moss & Leblond 1970, 1971). In minced muscle autografts, whilst myonuclei rapidly become pyknotic, ^3H -thymidine-labelled satellite cells remain viable and are retained beneath the relatively persistent basal laminae of myofibres (Snow 1977a, 1977b). Satellite cells also survive minced or whole muscle homografting, and are similarly the likely source of regeneration (Snow 1978, Schultz *et al.* 1988). Autografts of clonal cell cultures were used to demonstrate that isolated satellite-derived cells could participate in the repair and growth of myofibres *in vivo* (Lipton & Schultz 1979). In explants of minced ^3H -thymidine-treated muscles, labelled satellite cells were found to be the origin of proliferating MPC, and subsequently, differentiated myotubes (Trupin *et al.* 1982). The development of single myofibre isolation techniques provided the first definitive demonstration that in tissue culture, mouse (Rosenblatt *et al.* 1996), rat (Bischoff 1986) and human (Bonavaud *et al.* 2002) anatomically-defined satellite cells are capable of generating MPC and differentiated myofibres in the absence of any other proliferative phenotypes (Bischoff 1986, Rosenblatt *et al.* 1996, Bonavaud *et al.* 2002).

The definitive anatomical position of satellite cells (Mauro 1961) is necessarily lost by cells that fulfil their regenerative function in becoming activated and proliferating to generate differentiation-competent MPC which are outside the satellite cell niche. For this reason, although functional data is frequently attributed to satellite cells, in most cases, the link between satellite cell, MPC and regenerated myofibre is largely circumstantial (reviewed, Partridge 2003).

(b) Quiescence and activation

In growing muscles, satellite cells are metabolically and mitotically active as indicated by their abundant cytoplasm and numerous mitochondria (Schultz 1976), and their spontaneous incorporation of labels such as ^3H -thymidine (Moss & Leblond 1970, 1971, Snow 1977b, 1978) and BrdU (Schultz 1996). In uninjured adult muscles, myonuclei turn over slowly, at a rate of 1-2% per week (Schmalbruch & Lewis 2000), and satellite cells comprise <5% of total muscle nuclei (Bischoff 1994). Accordingly, adult satellite cells are metabolically inactive (Schultz 1976) and mitotically quiescent (Schultz *et al.* 1978). Phenotypic markers of quiescent satellite cells include Pax7 (Seale *et al.* 2000, Zammit *et al.* 2002, 2004a), another transcription factor, Sox8 (Schmidt *et al.* 2003), CD34 (Beauchamp *et al.* 2000), c-met (Cornelison & Wold 1997) and the adhesion protein M-cadherin (Irintchev *et al.* 1994, Kuschel *et al.* 1999). Single cell analysis failed to detect *Myf5* mRNA in quiescent satellite cells (Cornelison & Wold 1997). However, in the gene-targeted *Myf5^{nLacZ/+}* mouse (Tajbakhsh *et al.* 1996a), most quiescent satellite cells have detectable levels of Myf5- β -gal fusion protein (Beauchamp *et al.* 2000, Heslop *et al.* 2001). The C2C12 immortalised mouse myoblast cell line (Yaffe & Saxel 1977) is frequently used to model myogenesis. In the C2C12 cells, Myf5 protein is high in G₀ and transiently decreases during G₁ (Kitzmann *et al.* 1998).

Quiescent satellite cells express a truncated isoform of CD34 lacking an intracellular domain, which in the full length isoform, contains three potential phosphorylation sites. Satellite cell activation is characterised by a transient switch to expression of the full length isoform by alternative splicing. Full length CD34 is then rapidly down-regulated and neither isoform is expressed after the early stages of activation (Beauchamp *et al.* 2000). Proliferating C2C12 cells do not express CD34 protein. However, when differentiation is induced, a subset of cells form a reserve population which remains mononucleate, down-regulates expression of Myf5 and MyoD proteins, and expresses CD34 protein. These cells are usually seen in close association with differentiated myotubes and may be analogous to satellite cells (Beauchamp *et al.* 2000).

Quiescent satellite cells are activated *in vivo* in response to myofibre damage or denervation (Snow 1977a, 1977b, 1983, Schmalbruch & Lewis 2000, reviewed,

Bischoff 1994) and *in vitro*, on exposure to serum mitogens (Bischoff 1996, Zammit *et al.* 2002, 2004), HGF (Tatsumi *et al.* 1998, Sheehan *et al.* 2000, Anderson & Pilipowicz 2002), fibroblast growth factor (FGF) (Bischoff 1986) or nitric oxide (Anderson & Pilipowicz 2002). Satellite cell activation is initially characterised by upregulation of *Myf5* and *MyoD* mRNA and protein expression (Cornelison & Wold 1997, Cooper *et al.* 1999, Zammit *et al.* 2002, 2004a), which precedes mitotic activity as defined by expression of proliferating cell nuclear antigen (PCNA), or incorporation of BrdU into DNA precursors (reviewed, Sabourin & Rudnicki 2000). Activated satellite cells rarely express *MyoD* in the absence of *Myf5* (Zammit *et al.* 2002). In C2C12 cells, *MyoD* protein is absent in G₀ but peaks in mid-G₁ (Kitzmann *et al.* 1998).

In vivo, satellite cells undergo several rounds of proliferation to generate a pool of MPC (reviewed, Bischoff 1994, Sabourin & Rudnicki 2000). In tissue culture, it has been shown that the discrete populations of satellite cells associated with isolated single myofibres each have the proliferative competence to completely replace the parent myofibre within four days (Zammit *et al.* 2002). Satellite-derived cells can migrate to foci of damage within the same muscle (Schultz *et al.* 1985), or outwards to the periphery of the muscle to escape ischaemia (Schultz *et al.* 1988), but do not contribute to regeneration of adjacent damaged muscles in the absence of a physical bridge (Schultz *et al.* 1986). MPC grafts generate muscle that is mainly localised to the transplant site. However, several months after grafting, small numbers of donor-derived myofibres can be identified in adjacent muscles, suggesting that satellite cells have a limited inter-muscle migratory ability (Watt *et al.* 1987, Jockusch & Voigt 2003).

(c) Differentiation and fusion

Satellite cell-derived MPC commit to differentiation and exit the cell cycle (Andrés & Walsh 1996, Zammit *et al.* 2004a), before terminally differentiating and fusing into myofibres (Moss & Leblond 1971, Robertson *et al.* 1993, Rosenblatt *et al.* 1995). The early stage of commitment to differentiation is characterised by the expression of myogenin and *Mrf4* mRNA and protein (Smith *et al.* 1994, Yablonka-Reuveni & Rivera 1994, Cornelison & Wold 1997, reviewed, Chargé & Rudnicki 2003). Myogenin⁺ cells are capable of one or two further population doublings, before

exiting the cell cycle concomitant with the expression of p21 inhibitor (Andrés & Walsh 1996). MyoD is initially co-expressed with myogenin, but is later down-regulated (Fuchtbauer & Westphal 1992). *MyoD*^{-/-} muscles contain elevated numbers of morphologically-normal satellite cells. However, the inability of these cells to progress through the normal developmental program of MRF expression results in significant regenerative impairment (Megeney *et al.* 1996). The regulation of myoblast cell cycle progression is thought to be controlled by the winged helix transcription factor, myocyte nuclear factor (MNF) (Garry *et al.* 1997, 2000). Mice deficient in MNF have impaired muscle regeneration (Garry *et al.* 2000). M-cadherin protein is required for differentiation (Zeschnigk *et al.* 1995), but is down-regulated after fusion (Irintchev *et al.* 1994). Interference with M-cadherin protein expression prevents fusion of myoblasts *in vitro* (Zeschnigk *et al.* 1995). Like M-cadherin, Sox8 is expressed by quiescent satellite cells. However, Sox8 is down-regulated prior to differentiation, and fusion is inhibited by forced Sox8 expression. This suggests that Sox8 has a role in maintaining the proliferative competence of satellite cells (Schmidt *et al.* 2003).

Examination of regenerating skeletal muscles at the ultrastructural level has shown that fusion occurs between myoblast and myoblast, myoblast and myotube, myotube and myotube, and at later timepoints, myotube and more highly-differentiated myofibre (Robertson *et al.* 1990, 1993). Whilst mature myofibres are mainly peripherally nucleated, in the mouse, newly-regenerated myofibres can be identified by their distinctive central nucleation (Coulton *et al.* 1988). Rouger *et al.* (2004) recently provided evidence for the existence of two different populations of turkey myoblasts which fuse differently both *in vitro*, and *in vivo* after autografting of labelled cells. A subset of slowly-proliferating clones were found to fuse mainly with each other whereas highly-proliferative clones fused mainly with other mature myofibres (Rouger *et al.* 2004).

Similarly to embryonic muscle progenitors, the progeny of adult mouse satellite cells have been demonstrated to have intrinsic commitment to different patterns of MyHC expression. Satellite cells committed to slow myosin expression are preferentially associated with slow myofibres (Rosenblatt *et al.* 1996). In contrast, human satellite

cell-derived myoblasts are not committed to expressing particular MyHC isoforms (Bonavaud *et al.* 2001).

In vitro, satellite cells also have the ability to adopt alternative non-muscle fates and differentiate along adipogenic or osteogenic lineages (Asakura *et al.* 2001).

1.43 Evidence for resident MPC outside the satellite cell niche

The ability to exclude Hoechst dye defines the SP subset of bone marrow, a purified population of HSC (Goodell *et al.* 1996). A similar protocol has been used to obtain a Hoechst dye-excluding subset from skeletal muscle tissue, termed muscle SP (Gussoni *et al.* 1999, Jackson *et al.* 1999). In contrast the main population (MP) of Hoechst dye-retaining muscle cells, muscle SP has been demonstrated to have both myogenic and haematopoietic potential (Gussoni *et al.* 1999, Jackson *et al.* 1999, Asakura *et al.* 2002, McKinney-Freeman *et al.* 2003), and also has other characteristics which suggest it is distinct from the satellite cell compartment (Asakura *et al.* 2002).

Muscle SP is negative for expression of satellite cell markers such as desmin, Pax7 and Myf5, positive for expression of the satellite and endothelial cell marker CD34, and positive for expression of HSC markers such as Sca-1 and CD45 (Asakura *et al.* 2002, McKinney-Freeman *et al.* 2003), neither of which are expressed by anatomically-defined satellite cells (Zammit & Beauchamp 2001, Asakura *et al.* 2002). The haematopoietic activity of muscle SP is entirely localised to CD45⁺ cells (McKinney-Freeman *et al.* 2003). *In vitro*, the unsorted population is capable of both myogenic and haematopoietic differentiation, satellite cells of only myogenic differentiation, and muscle SP of spontaneous haematopoietic differentiation, and of myogenic differentiation only after co-culture with already-committed myogenic cells (Asakura *et al.* 2002). Whilst satellite cells cannot be grafted intravenously (Sampaloesi *et al.* 2003), muscle SP delivered via the vasculature can enter muscle tissue and undergo myogenic differentiation (Gussoni *et al.* 1999, Asakura *et al.* 2002).

Muscle SP has functional and phenotypic characteristics which suggest it forms a separate population to bone marrow SP. In competitive haematopoietic repopulation assays, muscle SP is 22 times less potent than bone marrow SP (McKinney-Freeman *et al.* 2003). Muscle SP is negative for expression of bone marrow SP antigens such as vascular endothelial growth factor-A and Tal-1 (Majka *et al.* 2003), and CD3, CD4, CD8 and c-kit (Benchouir *et al.* 2004).

The haematopoietic fraction of muscle SP has been shown to originate from bone marrow (Majka *et al.* 2003). However, a long-term study found that though bone marrow-derived cells are present in skeletal muscle, they do not make a significant contribution to the whole fraction of muscle SP. This implies that muscle SP is primarily derived from a tissue-resident source (Rivier *et al.* 2004). It has been demonstrated that a Hoechst dye-excluding SP population exists in state of dynamic equilibrium within the C2C12 immortal cell line (Benchouir *et al.* 2003). Similarly to both bone marrow SP and muscle SP, C2C12 SP expresses Sca-1 and CD34 (Benchouir *et al.* 2003). Significantly, however, it does not express canonical haematopoietic markers such as CD45 (Benchouir *et al.* 2003), which define the haematopoietic fraction of muscle SP (McKinney-Freeman *et al.* 2003). Taken together, the results of these studies (Majka *et al.* 2003, Rivier *et al.* 2004, Benchouir *et al.* 2004) imply that in principal, the haematopoietic and myogenic activities of muscle SP might reside in separate subpopulations with distinct origins. Recent studies by Rudnicki's group, however, suggest that muscle-resident CD45⁺ cells are not always restricted to the haematopoietic lineage. CD45⁺ Sca-1⁺ muscle-derived cells purified from uninjured muscles were found to be uniformly non-myogenic. But when CD45⁺ Sca-1⁺ cells were sorted from injured muscles, they expressed muscle-specific markers such as desmin and Pax7, and underwent spontaneous differentiation into myotubes. This suggests that damage induces the myogenic specification of a previously uncommitted CD45⁺ Sca-1⁺ population (Polesskaya *et al.* 2003). *Pax7*^{-/-} muscles have significantly reduced numbers of satellite cells, but still contain populations of CD45⁺ Sca-1⁺ cells (Seale *et al.* 2000). Myogenic specification of *Pax7*^{-/-} CD45⁺ Sca-1⁺ muscle-derived cells can be induced by viral infection with the *Pax7* gene, which *in vivo*, is sufficient to induce regeneration of new myotubes (Seale *et al.* 2004).

1.44 The contribution of circulatory cells to adult muscle

The idea that cells from the circulation could contribute to skeletal muscle was introduced by Bateson *et al.* (1967), who showed that there was a period of delay between the intravenous injection of ^3H -thymidine and the incorporation of label into regenerated myonuclei, implying that putative muscle precursors could acquire radioactive label outside the tissue, and then migrate into it (Bateson *et al.* 1967, commentary, Partridge 1998). The possibility that rapidly-cycling cells could have incorporated label, and then released it a few hours later to be reutilised by other cells, renders the authors' interpretation of the data equivocal (reviewed, Sloper & Partridge 1980). Later, however, Ferrari *et al.* (1998) provided an unequivocal demonstration of the integrity of the concept using bone marrow grafts derived from transgenic 3F-*nLacZ*-2E mice, in which nuclear β -gal reports expression of the skeletal muscle fibre-specific myosin light chain 3F gene (Ferrari *et al.* 1998, commentary, Partridge 1998). It was found that small numbers of bone marrow cells were incorporated into regenerating myofibres and expressed the transgene both when the graft was inserted directly into muscle tissue, and when it was delivered systemically (Ferrari *et al.* 1998). The results of numerous subsequent studies have supported this finding, demonstrating that bone marrow-derived cells can both contribute to differentiated muscle fibres (Bittner *et al.* 1998, Ferrari *et al.* 2001, Gussoni *et al.* 2002, Corti *et al.* 2002, LaBarge & Blau 2002, Fukada *et al.* 2002, Brazelton *et al.* 2003, Corbel *et al.* 2003, Camargo *et al.* 2003, Dreyfus *et al.* 2004, Shi *et al.* 2004) and enter the satellite cell compartment (Fukada *et al.* 2002, LaBarge & Blau 2002, Dreyfus *et al.* 2004). There have also been shown to be differences between different muscles in the frequency with which bone marrow-derived cells are incorporated (Brazelton *et al.* 2003). Whilst most of these data were obtained using mouse models, one study demonstrated the long-term persistence of donor-derived myofibres in a human muscular dystrophy patient who had received bone marrow transplantation (Gussoni *et al.* 2002). In mice, it has been shown that lethally-irradiated recipients haematopoietically reconstituted with single defined HSC can incorporate graft-derived cells into regenerating skeletal muscle fibres (Camargo *et al.* 2003, Corbel *et al.* 2003), and at lower frequency, non-regenerating fibres (Corbel *et al.* 2003), although there is little evidence that HSC can give rise to committed myogenic progenitors (Camargo *et al.* 2003). The rarity of such myogenic differentiation events

may explain why other workers have failed to identify myogenic activity in the haematopoietic fraction of human bone marrow (Shi *et al.* 2004), although myogenic potential has been identified in the whole population (Gussoni *et al.* 2002, Bossolasco *et al.* 2004) and its stromal fraction (Shi *et al.* 2004).

The observation that cells of the myeloid lineage can enter the satellite cell compartment is in fact not new; 25 years ago Trupin *et al.* (1979) reported the presence of macrophages under the basal lamina of myofibres in regenerating rat muscles, which they described as satellite cell 'mimics'. More recently, the finding that long-term exercise regimes augment numbers of graft-derived myofibres has been cited as evidence that bone marrow-derived satellite cells are capable of subsequent participation in regeneration (LaBarge & Blau 2002). However, it is also feasible that the frequency of bone marrow-derived myofibres increases over time as a result of further recruitment from the circulation, rather than by clonal expansion of graft-derived satellite cells. Myogenesis can occur concomitantly with angiogenesis after muscle tissue damage (Scholz *et al.* 2003). The very low incidence of bone marrow-derived myogenic activity reported by all workers (Ferrari *et al.* 1998, 2001, Gussoni *et al.* 2002, Corti *et al.* 2002, LaBarge & Blau 2002, Brazelton *et al.* 2003, Corbel *et al.* 2003, Camargo *et al.* 2003, Dreyfus *et al.* 2004, Shi *et al.* 2004), even in highly regenerative environments (Gussoni *et al.* 2002, Ferrari *et al.* 2001, Camargo *et al.* 2003, Corbel *et al.* 2003) accords well with the finding of a lineage tracking experiment which showed that HSC-derived skeletal muscle is generated stochastically through myeloid, rather than satellite cell or other proliferative MPC intermediates (Camargo *et al.* 2003, reviewed, Camargo *et al.* 2004b). However, on entry into muscle tissue, bone marrow-derived cells are reported to silence expression of CD45, implying a reduced commitment to the myeloid lineage (Rivier *et al.* 2004). And conversely, other recent studies have identified human CD45⁺ desmin⁺ bone marrow-derived cells which differentiate into muscle *in vitro* in the absence of other myogenic cell types (Bossolasco *et al.* 2004), and also human AC133⁺ (a haematopoietic cell surface antigen of unknown function) circulatory cells which generate muscle with relatively high efficiency after grafting, and may also give rise to satellite cells (Torrente *et al.* 2004). Similarly, a small proportion of mouse bone marrow cells has been shown to express the MRFs Myf5 and MyoD, and to undergo myogenic differentiation *in vitro* (Bhagavati & Xu 2004). This endogenous

commitment to the myogenic lineage (albeit of a small subset) implies that MPC can sometimes derive from bone marrow and that the process of myogenic recruitment, though inefficient, may occur via a physiological rather than merely stochastic process.

1.45 The origin of postnatally-renewed satellite cells

(a) Evidence that functional satellite cells are renewed from a tissue-resident source

Exposure of mouse muscles to high local doses of irradiation results in a long-term growth deficit (Wakeford *et al.* 1991, Quinlan *et al.* 1995) and sustained depletion of replication-competent satellite cells (Heslop *et al.* 2000). For this reason, though a detailed examination of the functional attributes of bone marrow-derived satellite cells is lacking, it seems unlikely that circulatory cells can generate precursors with any significant myogenic stem cell potential (commentary, Partridge 1998).

In young animals, following growth (Schultz 1996), and after the perturbation of injury and regeneration (Schultz 1984, Morlet *et al.* 1989, Reimann *et al.* 2000), satellite cell numbers are maintained, and muscle tissue retains the potential to respond to subsequent regenerative events (Schultz & Jaryszak 1985, Morlet *et al.* 1989, Luz *et al.* 2002). However, satellite cell numbers decline with age (Gibson & Schultz 1983, Kadi *et al.* 2004, Sajko *et al.* 2004), and the proliferative competence of MPC becomes diminished both with age (Sadeh 1988), and after repeated bouts of regeneration (Schultz & Jaryszak 1985). There is therefore good circumstantial evidence suggesting firstly, that functional satellite cells have a finite potential for renewal, and secondly, that they are derived from a tissue-resident source, either outside or within their own niche (**Fig. 1.2**).

(b) Renewal from resident cells located outside the satellite cell compartment

A study by Heslop *et al.* (2000) showed that proliferation-competent satellite cells are not present in single myofibre cultures derived from mouse muscles treated with 18 Gy irradiation. However, when irradiated muscles are damaged and then allowed to regenerate, some single myofibres derived from regenerated muscles have associated satellite cells capable of proliferation *in vitro*. Muscles exposed to 25 Gy irradiation

and subsequently damaged have a (very) limited capacity to regenerate, but do not harbour replication-competent satellite cells (Heslop *et al.* 2000). In addition, it has been shown that a minority of turkey MPC are resistant to the effects of 25 Gy irradiation, and after an extended period in culture, can enter the cell cycle and undergo proliferation (Mozdziak *et al.* 1996). The findings of Heslop *et al.* (2000), which suggest that satellite cells are derived from a source outside the compartment, but within the tissue, were cited as evidence that CD45⁺ Sca-1⁺ putative muscle stem cells are tissue-resident, rather than derived from the circulation (Polesskaya *et al.* 2003). CD45⁺ Sca-1⁺ cells spontaneously express the satellite cell determinant, Pax7, after damage (Polesskaya *et al.* 2003), and moreover can be induced to undergo myogenic commitment by infection with Pax7 (Seale *et al.* 2004). Hypothetically, CD45⁺ Sca-1⁺ cells could therefore be a source of functional satellite cells, and perhaps be derived from the same population as the radiation resistant cells. However, the lack of data describing the phenotypic characteristics of radiation-resistant cells (Heslop *et al.* 2000) and the anatomical location of the progeny of CD45⁺ Sca-1⁺ cells (Polesskaya *et al.* 2003, Seale *et al.* 2004) make any such relationship unassessable. Nonetheless, the finding that Sca-1⁺ SP cells exist in a state of dynamic equilibrium within the C2 line (Benachouir *et al.* 2003) suggests that satellite cells could periodically exit their anatomical niche and adopt a Sca-1⁺ (non-satellite cell) phenotype.

(c) Evidence that satellite cells self-renew

An early study found that *in vivo*, some of the progeny of ³H-thymidine-labelled satellite cells did not fuse into myofibres, but retained their satellite cell status (Moss & Leblond 1971). Several studies have shown that grafts of MPC into mouse muscles both generate differentiated tissue, and maintain a reserve population of undifferentiated MPC (Yao & Kurachi 1993, Morgan *et al.* 1994, Blaveri *et al.* 1999, Gross & Morgan 1999, Heslop *et al.* 2001). Two studies showed that MPC could generate anatomically-defined satellite cells (Blaveri *et al.* 1999, Heslop *et al.* 2001), either when the donor cells were heterogeneous primary neonatal myoblasts (Heslop *et al.* 2001), or an immortalised satellite cell-derived cell line (Blaveri *et al.* 1999), the latter showing that the progeny of a single satellite cell can adopt divergent fates. It has been demonstrated that transplanted conditionally-immortalised MPC maintain some myogenic potential after serial passage through several muscles, with intervals

of *in vitro* expansion (Morgan *et al.* 1994). However, the somewhat limited ability of transplanted MPC to respond to regenerative events *in vivo* (Gross & Morgan 1999) implies that cells cannot undergo significant clonal expansion outside the tissue culture environment. In the absence of immortalisation, the ability of a transplanted satellite-derived cell to undergo self-renewal *in vivo* is not known.

In tissue culture, clonal populations of MPC also adopt divergent fates on differentiation (Barroffio *et al.* 1996, Yoshida *et al.* 1998, Zammit *et al.* 2004a). Upon induction of differentiation by serum deprivation, most C2C12 cells initiate expression of myogenin, withdraw from the cell cycle, terminally differentiate and fuse into myotubes. However, a subset down-regulate expression of Myf5 and MyoD, but do not express myogenin or undergo fusion, and retain the ability to proliferate when re-stimulated with serum (Yoshida *et al.* 1998). These reserve cells also express the satellite cell marker, CD34 (Beauchamp *et al.* 2000). Similarly, primary myoblasts isolated from human muscle tissue generate a reserve population on differentiation, which can, however, be re-stimulated to proliferate, differentiate and yield up to four further cohorts of reserve cells (Baroffio *et al.* 1996). Recently, it has been demonstrated that a similar phenomenon occurs with satellite cells associated with isolated myofibres in suspension culture (Zammit *et al.* 2004a). In response to serum stimulation, satellite cells initially proliferate to generate small clonal populations. Within each clone, most cells commit to differentiation as defined by expression of myogenin and cell cycle withdrawal, but a minority are recalcitrant to differentiation and retain robust expression of Pax7 protein (Zammit *et al.* 2004a). Satellite cells derived from *Pax7*^{-/-} mice express MyoD, proliferate and differentiate normally in tissue culture. However, numbers of satellite cells in *Pax7*^{-/-} mice decline as animals reach maturity (Oustanina *et al.* 2004). Taken together, the data from these two studies suggest that the satellite adult satellite cell pool might be maintained by a process of self-renewal of which *Pax7* is a key regulator (Oustanina *et al.* 2004, Zammit *et al.* 2004a).

(d) Evidence that satellite cells are renewed by a stem cell subset

Studies of isolated single myofibres have found that the satellite cell compartment is phenotypically heterogeneous. Beauchamp *et al.* (2000) showed that while the majority of satellite cells express Myf5, CD34 and M-cadherin, about 20% do not express any of these markers. It has been hypothesised that these negative cells may represent a more primitive, stem cell-like population which functions to renew the compartment (Beauchamp *et al.* 2000, reviewed, Zammit & Beauchamp 2001). On exposure to serum, 98% of satellite cells in single myofibre preparations synchronously activate and express MyoD protein; however, the remaining 2% appear to resist activation (Zammit *et al.* 2002). Whether these cells represent a deeply-quiescent stem cell subset, or are merely replication-incompetent, remains unclear.

Studies of regeneration *in vivo* have suggested that satellite cells also exhibit functional heterogeneity (Rantanen *et al.* 1995, Schultz 1996, Rouger *et al.* 2004). Rantanen *et al.* (1995) found that while some stem cell-like satellite cells underwent proliferation in response to injury, other satellite cells upregulated myogenin expression within eight hours, implying a prior commitment to differentiation. In the muscles of growing rats, 80% of the satellite cell compartment was found to be labelled after five days of continuous BrdU infusion (Schultz 1996). The remaining 20% represented slower-cycling subset which incorporated BrdU more slowly, and was speculated to continuously renew the main population (Schultz 1996). Similarly, Rouger *et al.* (2004) have shown that proliferative heterogeneity exists between clones of myoblasts isolated from turkey muscles. After eleven days in culture, 50% of clones were found to give rise to ≥ 64 nuclei and were termed slowly-proliferating clones. 8% of clones gave rise to ≥ 256 nuclei and were termed highly-proliferative clones. The proliferative characteristics of clones were maintained both *in vitro* after co-culture with heterogeneous primary cells and *in vivo* after autografting. *In vivo*, slowly-proliferating clones were thought to give rise satellite cells more frequently than highly-proliferative clones (Rouger *et al.* 2004), though since neither phenotypic markers nor electron microscopy were employed, this observation is not robust. It is conceivable that the slow-cycling subsets identified by Schultz (1996) and Rouger *et al.* (2004) may correspond to the phenotypically unmarked population identified in the mouse satellite cell compartment by Beauchamp *et al.* (2000).

An alternative interpretation of these data is that rather than forming distinct subsets, all satellite cells are essentially similar but are behaviourally and phenotypically asynchronous. Whilst the majority of HSC are quiescent at any one timepoint, they are recruited back into the cell cycle at regular intervals (Bradford *et al.* 1997, Cheshier *et al.* 1999). Myoblast cell lines adopt divergent fates upon differentiation (Yoshida *et al.* 1998, Blaveri *et al.* 1999) and generate subsets which are slowly-proliferating *in vitro*, but nevertheless have the potential to undergo extensive proliferation *in vivo* (Beauchamp *et al.* 1999). In addition, C2C12 cells can generate reversible SP (Benchouir *et al.* 2003) and tumorigenic (Morgan *et al.* 2002) phenotypes, demonstrating that extreme behavioural diversity is possible within a single myoblast clone.

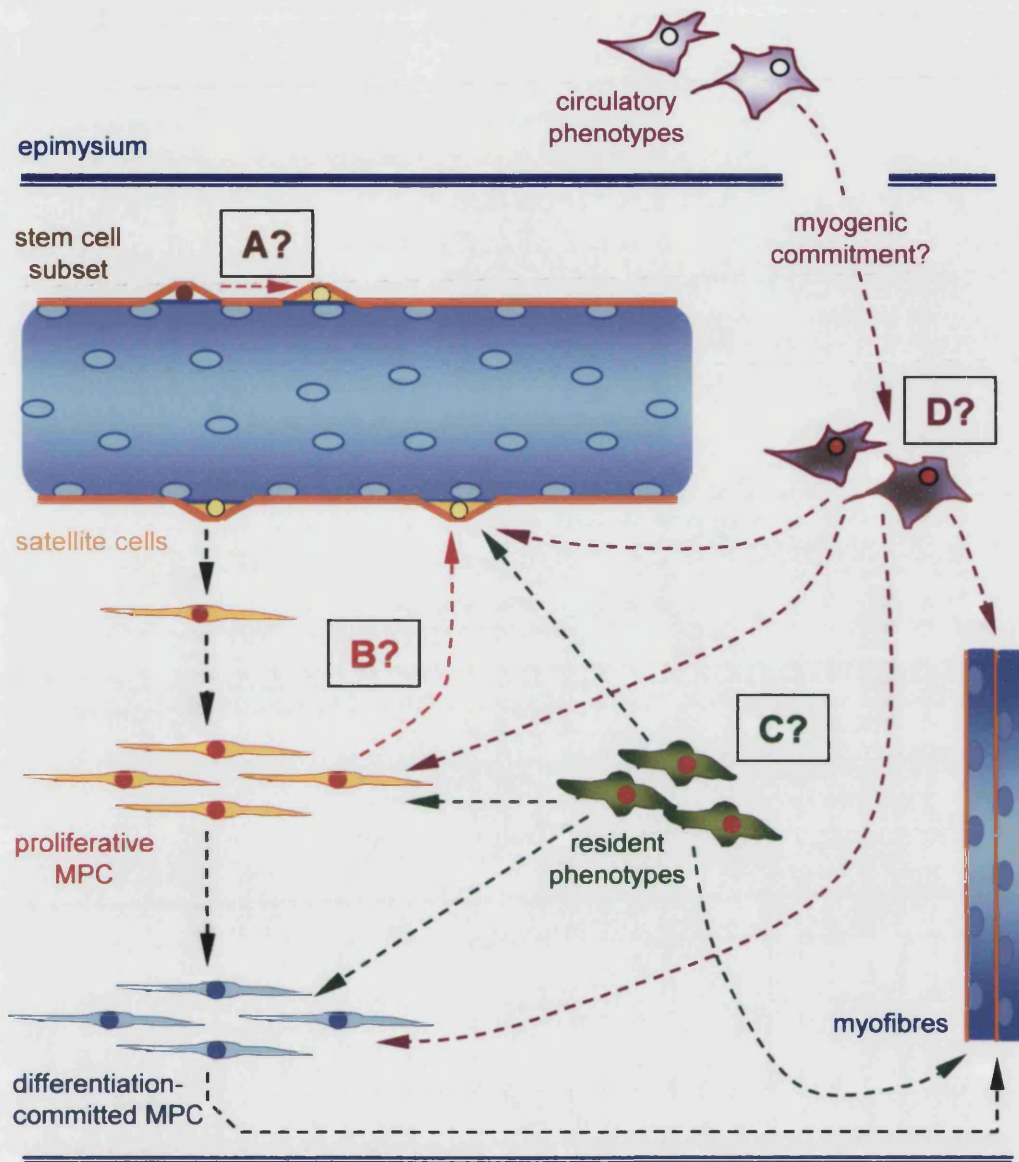


Fig. 1.2. The origin of postnatally-renewed satellite cells. The satellite cell compartment is maintained throughout adult life, but its major source of renewal has not been unequivocally established. The phenotypic (Beauchamp *et al.* 2000) and behavioural (Schultz 1996) heterogeneity of satellite cells predicts the existence of a stem cell-like subset which may function to renew the compartment (A). *In vitro* (Baroffio *et al.* 1996, Zammit *et al.* 2004a) and *in vivo* (Heslop *et al.* 2001), myoblasts maintain reserve populations upon differentiation, suggesting that satellite cell progeny can return to quiescence and thereby self-renew (B). Satellite cells could also be renewed by other resident phenotypes such as radiation-resistant MPC (Heslop *et al.* 2000), muscle SP (Asakura *et al.* 2002) or CD45⁺ Sca-1⁺ cells (Polesskaya *et al.* 2003) (C), or alternatively, by a circulatory phenotype (LaBarge & Blau 2002, Dreyfus *et al.* 2004) (D). It is not known whether these alternative phenotypes generate muscle via committed intermediate progenitors, or whether they fuse directly into myofibres without prior myogenic commitment (C, D).

1.5 Skeletal muscle myopathy

1.51 Myopathies involving the dystrophin-glycoprotein complex

The dystrophin-glycoprotein complex (DGC) is a large complex of transmembrane and membrane-associated proteins that connects the subsarcolemmal cytoskeleton of myofibres with the extracellular matrix (reviewed, Rando 2001, Durbeej & Campbell 2002). The major components of the DGC are dystrophin (Campbell & Kahl 1989), the α - and β - dystroglycans (Ervasti *et al.* 1990), the α -, β -, δ - and γ -sarcoglycans (Ibraghimov-Beskrovnaya *et al.* 1992), α -dystrobrevin (Grady *et al.* 1999), the $\alpha 1$ -, $\beta 1$ and $\beta 2$ -syntrophins (Adams *et al.* 1993), sarcospan (Crosbie *et al.* 1997), dysferlin, calveolin-3 and the $\alpha 5$ - and $\alpha 7$ -integrins (reviewed, Durbeej & Campbell 2002) (*Fig. 1.2*). The peripheral membrane protein α -dystroglycan binds to laminin-2 in the basal lamina, and in the extracellular matrix, to the heparan sulphate proteoglycans perlecan and agrin (which are also present in the basal lamina) and the chondroitin sulphate proteoglycan, biglycan. In addition, α -dystroglycan is associated with the extracellular domain of the transmembrane protein β -dystroglycan. β -dystroglycan binds to dystrophin which binds to the cytoskeletal protein, actin. Whilst the function of the DGC is not fully understood, it is believed have a structural role, protecting muscle cells from contraction-induced injuries (reviewed, Rando 2001, Durbeej & Campbell 2002). It is also involved in extracellular signalling (reviewed, Rando 2002). Mutations which cause disruption of the DGC component proteins lead to muscular dystrophies of varying severity (reviewed, Durbeej & Campbell 2002). In two examples, mutations in the gene encoding β -sarcoglycan cause the 2E form of limb-girdle muscular dystrophy (LGMD) (Lim *et al.* 1995, Bönneman *et al.* 1995), and mutations in the gene encoding dystrophin lead to DMD and its milder allelic form, Becker's muscular dystrophy (BMD) (Hoffman *et al.* 1987). Pathologically similar dystrophies are also generated by disruption of the matrix proteins to which the DGC binds. Mutations in the gene encoding laminin $\alpha 2$ chain are the genetic basis of congenital muscular dystrophy (CMD) (Sunada *et al.* 1994, Xu *et al.* 1994) (*Fig. 1.3*).

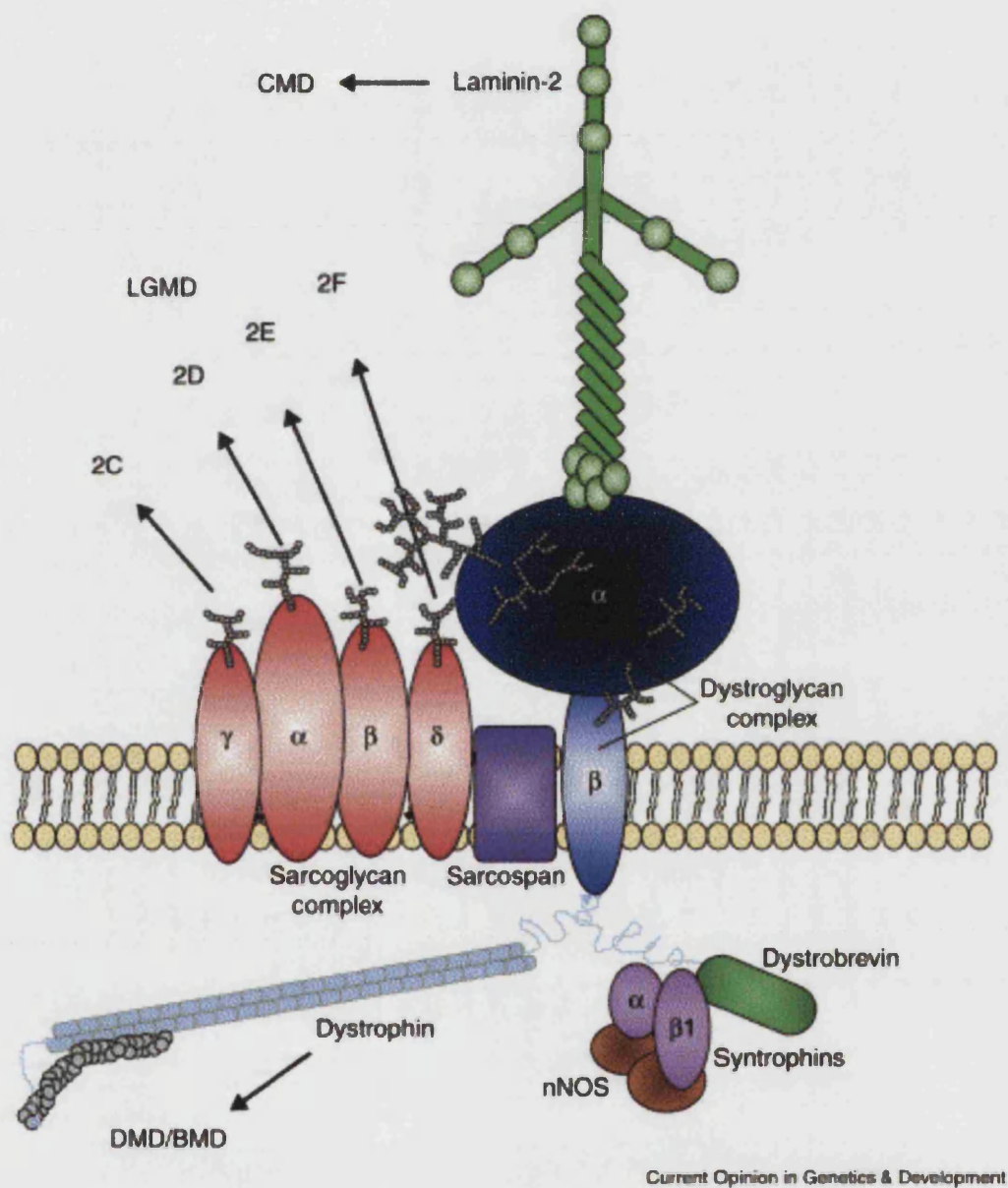


Fig. 1.3. The dystrophin-glycoprotein complex. Reproduced from Durbeej & Campbell (2002).

1.52 DMD

DMD is a lethal X-linked primary myopathy (Dubowitz 1985, Morgan *et al.* 1989, Hoffman & Schwartz 1991) characterised by the near-absence of dystrophin protein in skeletal muscles (Bonilla *et al.* 1988, Hoffman *et al.* 1988). It was first described in detail in the 19th Century, most notably by the physician G.B.A. Duchenne (Duchenne 1868 cited in Bogdanovich *et al.* 2004, Dubowitz 1992, reviewed, Tyler 2003). DMD is both the most severe and the most common of the myopathies involving the DGC, affecting one in every 3,500 live male births (Hoffman & Schwartz 1991, Dubowitz 1992).

In humans, the functional significance of dystrophin protein in skeletal muscles is demonstrated by the acute pathology resulting from its absence (Hoffman *et al.* 1988). Muscle histology in DMD patients is almost normal before the onset of clinical symptoms of at 3-5 years of age. The preliminary stage of the disease is characterised by the presence of focal groups of necrotic myofibres, muscle hypertrophy and abnormally high levels of muscle creatine kinase, an indicator of ongoing degeneration. In the second, pathological phase, repeated cycles of degeneration exhaust the regenerative capacity of skeletal muscle tissue (Dubowitz 1985, Dubowitz 1992, Rafael *et al.* 1997, Decary *et al.* 2000) and fibrotic mechanisms cause the progressive replacement of contractile muscle tissue with collagenous connective tissue (Dubowitz 1985, Dubowitz 1992, Rafael *et al.* 1997). This process results in severe joint contractures, loss of ambulation at around 10-12 years of age and respiratory problems, the culmination of which usually cause death during the third decade of life (Dubowitz 1985, Dubowitz 1992). The absence of dystrophin protein in the heart and brain also results in cardiomyopathy (Cziner & Levin 1993, Finsterer & Stollberger 2003) and cognitive impairment (Anderson *et al.* 2002, Wicksell *et al.* 2004).

1.53 Animal models of DMD

(a) Homologues of dystrophin and DMD in non-human species

Homologues of the human *dystrophin* gene (Hoffman *et al.* 1987a, 1987b) have been identified not only in vertebrates including mice (Hoffman *et al.* 1987a, 1987b), dogs (Cooper *et al.* 1988) and zebrafish (Bolanos-Jimenez *et al.* 2001) but also in

invertebrate models such as the fruit fly *Drosophila melanogaster* (Neumann *et al.* 2001) and the nematode worm *Caenorhabditis elegans* (Bessou *et al.* 1998). This conservation is indicative of the species-wide functional significance of dystrophin protein which is further demonstrated by the similarly universal, though differential, pathological reaction to its mutation or absence (reviewed, Collins & Morgan 2003). Whilst the effects of dystrophin deficiency in humans are profoundly debilitating from an early age, they are not recapitulated in the C57Bl/10-*mdx* (*mdx*) mouse model (Bulfield *et al.* 1984) which, despite being a genetic and biochemical homologue of DMD (Hoffman *et al.* 1987a, 1987b), has a normal lifespan and muscle pathology which does not become debilitating until old age (Dangain & Vrbova 1984, Tanabe *et al.* 1986, Cullen & Jaros 1988, Dubowitz 1992, reviewed, Partridge 1997). This species-specific response to dystrophin deficiency is informative regarding the function of the protein, suggesting that the disease state occurs as a consequence of secondary pathological mechanisms partly dependant on the host environment (Cullen & Jaros 1988, reviewed, Partridge 1997).

(b) The *mdx* mouse

Before the identification of the *dystrophin* locus in 1987 (Hoffman *et al.* 1987), several unauthenticated models of murine dystrophy were employed in DMD research, on the basis of their pathological similarities to the human disease. The most significant of these were the C57Bl/6 J-dy2J (*dy/dy*) mouse (Meier & Southard 1970) and the *mdx* mouse (Bulfield *et al.* 1984). The genetic basis of *dy/dy* murine dystrophy was eventually found to be a mutation in the gene encoding laminin M-chain (Sunada *et al.* 1994, Xu *et al.* 1994), showing it to be a genetic model of human CMD. With the identification of the *dystrophin* gene, however, it was revealed that human DMD and murine *mdx* dystrophy have a common locus (Hoffman *et al.* 1987a, 1987b).

Mdx is a spontaneous mutant that was identified within a wild-type C57Bl/10 colony from its elevated concentration of plasma creatine kinase (Bulfield *et al.* 1984). Creatine kinase peaks between two and eight weeks of age, during which period muscle pathology also becomes most pronounced and is characterised by the appearance of numerous necrotic foci and newly-regenerated centrally-nucleated myofibres (Tanabe *et al.* 1986, Coulton *et al.* 1988, reviewed, Partridge 1997). *Mdx*

pathology is thought to model DMD most closely between weeks three and four, during which time several cycles of degeneration and regeneration occur (Dangain & Vrbova 1984, Tanabe *et al.* 1986, reviewed, Partridge 1997). After this period, tissue efficiently regenerates and only mild myopathy persists in most muscles (Tanabe *et al.* 1986), although more severe pathology again becomes evident from 15 months onwards (Pastoret & Sebillé 1993). Regeneration is accompanied by myofibre hypertrophy, with *mdx* muscles having significantly greater masses than wild-type equivalents (Marshall *et al.* 1989, Pastoret & Sebillé 1993, Lynch *et al.* 2001). Though less pronounced than in DMD, fibrosis is also a feature of *mdx* myopathy, with collagen accumulation characteristic of affected muscles (Bulfield *et al.* 1984, Marshall *et al.* 1989, Morrison *et al.* 2000). The pathological response of different muscles is surprisingly distinct, with some, such as the masseter (Muller *et al.* 2001) and extraocular muscles (Karpati & Carpenter 1986, Karpati *et al.* 1988), being almost completely spared, and others, such as the diaphragm and gastrocnemius, affected severely in older animals (Stedman *et al.* 1991, Muller *et al.* 2001, Coirault *et al.* 2003). The diaphragm in particular has acute pathology comparable to that seen in the limb muscles of human DMD patients, and by age 2 years, has only 13.5% of the strength of the wild-type (Stedman *et al.* 1991, Muller *et al.* 2001). Before the onset of pathology at about 2 weeks of age, there is evidence that hindlimb muscles such as the EDL are insensitive to acute mechanical injury (Grange *et al.* 2002). However, in mature animals, not only the diaphragm (Stedman *et al.* 1991) but relatively non-pathological hindlimb muscles such as the TA are more susceptible to contraction-induced injury than in the wild-type (Dellorusso *et al.* 2001). *Mdx* muscles exhibit hypertrophy that is not solely attributable to fibrosis (Pastoret & Sebillé 1993), and has been suggested as the reason for excised juvenile EDL and soleus muscles showing no decrease in power output compared to wild-type muscles (Lynch *et al.* 2001). However, *mdx* mice do not adapt to voluntary exercise as well as wild-type controls, showing little increase in hindlimb muscle strength or decrease in fatigability over time (Carter *et al.* 1995); consequently, even moderate exercise may accelerate the course of the disease (De Luca *et al.* 2003).

It has been postulated that *mdx* pathology is moderated by the partial functional replacement of dystrophin by the homologous protein utrophin (Rybakova *et al.* 2002). Mice which are null for both dystrophin and utrophin (*mdx/utrn*^{-/-}) have an

acute phenotype and exhibit certain characteristics of human DMD which are not seen in the *mdx* mouse, including severe growth retardation, spinal curvature, joint contraction and premature death (Deconinck *et al.* 1997). In addition, it has been shown that early expression of utrophin in *mdx* mice can ameliorate dystrophic pathology to some extent (Squire *et al.* 2002).

There is evidence that dystrophic pathology such as that which develops in the later stages of *mdx* myopathy is in part attributable to the failure of the regenerative process (Sadeh *et al.* 1985, Schultz & Jaryszak 1985, Bockhold *et al.* 1998, Heslop *et al.* 2000, Reimann *et al.* 2000, Luz *et al.* 2002, Earnshaw *et al.* 2002). Although *mdx* muscle fibres initially develop normally, the differentiation of regenerated fibres is compromised (Earnshaw *et al.* 2002). Studies of cultured single myofibres by Bockhold *et al.* (1998) found that the numbers of proliferating satellite cells migrating from each fibre decline with age in both wild-type and *mdx* isolates. In the wild-type this decline is linear, and though by 550 d the satellite cell population is substantially depleted, virtually all emanating cells are of the myogenic lineage as defined by expression of the intermediate filament protein desmin. In *mdx* isolates the number of proliferative satellite cells increases with age up to 50 d, when it is three-fold greater than wild-type controls. Thereafter numbers of myogenic cells decline, but never recede below wild-type values. Significantly, however, there is an age-related increase in the number of non-myogenic cells, probably fibroblasts, which derive from cultured *mdx* myofibres. The results of this study therefore suggested that it is the combination of excess fibrinogenesis with normal age-related myogenic satellite cell decline, rather than myogenic cell decline alone, which is responsible for regenerative failure in muscular dystrophies (Bockhold *et al.* 1998). Nevertheless, murine *mdx* myopathy is at best an imprecise model of its human counterpart DMD (Dangain & Vrbova 1984, Tanabe *et al.* 1986, Cullen & Jaros 1988, reviewed, Partridge 1997). When wild-type rodent or *mdx* muscles are subjected to repeated rounds of damage by the myotoxic agent bupivacaine (Sadeh *et al.* 1985, Schultz & Jaryszak 1985, Luz *et al.* 2002), or to cryodamage (Reimann *et al.* 2000) their regenerative capacity is substantially compromised, suggesting that as in human DMD patients, the satellite cell compartment of rodents can be depleted with sufficient pressure. A study by Heslop *et al.* (2000) found that numbers of radiation-resistant muscle stem cells, a postnatal source of satellite cells, were greatly reduced in older

mdx mice compared to wild-type controls. Taken together, these studies suggest that although there is no overt difference in absolute numbers of satellite cells (Bockhold *et al.* 1998, Reimann *et al.* 2000) their potential for continued renewal is depleted (Heslop *et al.* 2000), and that *mdx* muscle tissue therefore manifests physiologically similar (though less advanced) regenerative exhaustion to DMD muscle.

(c) Canine and feline models

Spontaneous mutations of the *dystrophin* gene resulting in X-linked muscular dystrophy have been identified in several breeds of domestic dog, including the Golden Retriever (Cooper *et al.* 1988), the Rottweiler (B. Cooper cited in Partridge 1997) and the German short-haired pointer (Schatzberg *et al.* 1999). The Golden Retriever muscular dystrophic (GRMD) dog is the best characterised of these (Cooper *et al.* 1988).

In contrast to both *mdx* and DMD myopathies, GRMD pathogenesis manifests *in utero* with the development of lingual muscle lesions (Valentine *et al.* 1988, Nguyen *et al.* 2002), and neonatal GRMD puppies exhibit extensive necrosis of limb, trunk and neck muscles (Nguyen *et al.* 2002). Creatine kinase levels continue to increase steadily from birth onwards, reaching a plateau at 100X normal by six to eight weeks of age (Valentine *et al.* 1988), concurrent with the time of postnatal muscle maturation in dogs (Cozzi *et al.* 2001). Transforming growth factor- β 1 levels are also elevated and are thought to be involved in the initiation of fibrosis (Passerini *et al.* 2002). Severe fibrosis and contractures in joints such as the tarsus develop by 6 months of age (Valentine *et al.* 1988, Kornegay *et al.* 1994). Similarly to DMD patients, young GRMD dogs frequently die from cardiac or respiratory failure, though some survive to reach several years of age (Nguyen *et al.* 2002).

In cats, dystrophin deficiency results in hypertrophic feline muscular dystrophy (HFMD) (Kohn *et al.* 1993). As its name suggests, a defining feature of HFMD myopathy is pronounced muscle hypertrophy, which can culminate in lethal oesophageal obstruction and kidney failure (Kohn *et al.* 1993, Gaschen & Burgunder 2001). Although HFMD skeletal muscle undergoes repeated rounds of degeneration and regeneration, it does not develop the debilitating fibrosis that is characteristic of

DMD and GRMD (Kohn *et al.* 1993, Gaschen & Burgunder 2001), and is therefore closer to *mdx* dystrophy than its human counterpart.

(d) Non-mammalian models

Small animal models of DMD have been developed in the zebrafish (Guyon *et al.* 2003, Bassett *et al.* 2003, reviewed, Basset & Currie 2003) and the nematode *C. elegans* (Bessou *et al.* 1998). Zebrafish are attractive as models of myopathy because they have a high skeletal muscle content and express orthologues of most human DGC proteins which also have similar membrane localisation (Chambers *et al.* 2001, Guyon *et al.* 2003). Gene-targeting technologies are not currently available, but equivalent experiments can be carried out using oligonucleotide analogues (morpholinos) which disrupt translation of specific mRNA transcripts. This technique has been used to create dystrophin-deficient zebrafish which have an unstable DGC, bent morphology and lower activity (Guyon *et al.* 2003). In addition, a mutation called *sapje* in the zebrafish *dystrophin* orthologue has been shown to be responsible for a lethal embryonic phenotype attributed to the failure of muscle end attachments (Bassett *et al.* 2003).

The nematode worm *C. elegans* moves by contraction of longitudinal striated muscles which express a *dystrophin* orthologue called *dys-1* (Bessou *et al.* 1998), that interacts with other DGC proteins (Gieseler *et al.* 1999, Chamberlain & Benian 2000). Mutations of *dys-1* result in hypercontraction, increased sensitivity to the neurotransmitter acetyl choline, and hyperactivity (Bessou *et al.* 1998).

1.54 Myoblast transfer therapy

Haematopoietic reconstitution by transplantation of bone marrow cells has been used with some success as a treatment for disorders of the lymphohaematopoietic system (Faber-Langendoen *et al.* 1993, Cavazzana-Calvo *et al.* 2000). The rationale behind myoblast transfer therapy (MTT) is that the biochemical and pathological defects in DMD patients could be similarly treated by reconstitution of affected muscles with grafts of normal donor or genetically-corrected host MPC (reviewed, Smythe *et al.* 2001, Partridge 2002b, 2003).

The experiments of Studitsky and Carlson established the principle that minced muscle derived from one animal could regenerate new functional muscle tissue when grafted into the muscle bed of a second animal (Studitsky 1964, Carlson 1968, 1970). It was subsequently shown that isolated populations of muscle cells could also participate in regeneration after grafting directly into the muscles of rodents (Jones 1979, Lipton & Schultz 1979, Watt *et al.* 1982, Law *et al.* 1988). The ability of grafted cells to fuse into host myofibres (Partridge *et al.* 1978) was exploited as a means of inserting foreign genes into host muscle tissue (Watt *et al.* 1984a, Morgan *et al.* 1988), including the normal *dystrophin* gene in the muscles of *mdx* mice (Partridge *et al.* 1989). Importantly, grafts of normal MPC were found to generate myofibres expressing membrane-localised dystrophin protein, thus locally ameliorating both the genetic and biochemical defects (Partridge *et al.* 1989). It was subsequently also shown that normal muscle cell isolates grafted into the dystrophic muscles of *dy/dy* mice can restore expression of the absent laminin $\alpha 2$ protein (Vilquin *et al.* 1996).

The promising results of early animal studies were the inspiration behind clinical trials of MTT as a therapy for DMD (Law *et al.* 1991, 1992, Gussoni *et al.* 1992, Karpati *et al.* 1993, Tremblay *et al.* 1993, Mendell *et al.* 1995, Miller *et al.* 1997), and later, BMD (Neumeyer *et al.* 1998). The majority of these trials reported that MTT did not result in the restoration of functionally significant amounts of dystrophin protein expression (Gussoni *et al.* 1992, Karpati *et al.* 1993, Tremblay *et al.* 1993, Mendell *et al.* 1995, Miller *et al.* 1997, Neumeyer *et al.* 1998), though unexpectedly, administration of the immunosuppressive agent cyclosporine was found to augment muscle strength in treated boys (Miller *et al.* 1997). Later re-analysis of 6 month post-MTT muscle biopsies from one trial, using a fluorescence *in situ* hybridisation method, revealed that although a few myoblasts had persisted in all muscles, many cells had failed to differentiate into functional tissue (Gussoni *et al.* 1997). A major factor in the failure of MTT is likely to have been the substantial immune reaction elicited against the transplanted cells, despite careful selection of histocompatible donors (Huard *et al.* 1992, Roy *et al.* 1993). Beneficial effects from MTT were reported by one group (Law *et al.* 1991, 1992), but these studies omitted to control for the potential therapeutic effects of cyclosporine, and in subsequent independent verification no donor-derived dystrophin could be identified in patient muscle biopsies (Partridge *et al.* 1998).

The apparent lack of success of clinical trials of MTT could be at least partly attributable to the extreme disparity between the *mdx* mouse model and the human DMD patient (reviewed, Partridge 1997, 2002b). Apart from other obvious innate differences such as size, muscle formation in mouse models is usually optimised by prior treatment of the graft site with irradiation (Morgan *et al.* 1990, Kinoshita *et al.* 1994, Gross *et al.* 1999), cryodamage (Morgan *et al.* 1987, Wernig *et al.* 1991, Irintchev *et al.* 1997) or myotoxins (Huard *et al.* 1994, Kinoshita *et al.* 1994, Pye *et al.* 2004), none of which are feasible for use in human patients. In addition, many of the animal studies of MTT were carried out in immunodeficient hosts, such as *mdx* mice bred onto a nude background (Partridge *et al.* 1989), or the severe combined immune deficient (SCID) mouse (Huard *et al.* 1994). Studies of MTT in immunocompetent mice found that grafted myoblasts elicited a robust immune response as indicated by the infiltration of lymphocytes, macrophages, granulocytes and natural killer cells (Guerrette *et al.* 1995, Irintchev *et al.* 1995, Wernig *et al.* 1995, Hodgetts *et al.* 2000, Skuk *et al.* 2002), even when, as in human MTT trials, the donor cells were matched for host histocompatibility (Wernig & Irintchev 1995). As well as causing massive and rapid donor cell death (Fan *et al.* 1996, Hodgetts *et al.* 2000), the process of graft rejection has been shown to be damaging to the host tissue (Irintchev *et al.* 1995, Wernig & Irintchev 1995). The immunosuppressive drugs Cyclosporin A (Watt *et al.* 1984b, Irintchev *et al.* 1995) and FK506 (Kinoshita *et al.* 1994) improve the success of MTT in immunocompetent mice; FK506 has also been used to support efficient MTT in monkeys (Kinoshita *et al.* 1996, Skuk *et al.* 2000).

It has been demonstrated that the majority of myoblasts rapidly die following transplantation, irrespective of whether the host is immunocompetent or immunocompromised (Fan *et al.* 1996, Beauchamp *et al.* 1999, Hodgetts *et al.* 2000). A small subset survives to subsequently proliferate and expand the pool of differentiation-competent MPC (Beauchamp *et al.* 1999, Cousins *et al.* 2004). Beauchamp *et al.* (1997) developed a model system whereby male cells were labelled with ¹⁴C-thymidine and grafted into female host mice. Y chromosome was used as a conserved marker to quantify the amount donor genome present, and ¹⁴C scintillation counts were used to estimate cell survival (Beauchamp *et al.* 1997). This system was used to show that donor-derived muscle originates from a 'stem cell-like'

subpopulation of cells that in tissue culture, are divide slowly and therefore do not incorporate radioactive label, but after grafting undergo extensive proliferation (Beauchamp *et al.* 1999). Similarly, labelling of primary cultures with unique retroviral inserts showed that muscle regeneration from grafted myoblasts is oligoclonal (Cousins *et al.* 2004). Unlike mouse cells (Hauschka *et al.* 1979, Morgan *et al.* 1994) human myoblasts do not form cell lines, and have a finite proliferative capacity ('Hayflick's limit'), undergoing senescence after about twenty population doublings (reviewed, Di Donna *et al.* 2000). It is possible that the sometimes very extensive expansion of cells prior to MTT in DMD patients (Law *et al.* 1992) resulted in a transplanted population that was already close to senescence, and therefore incapable of proliferation equivalent to that of the myogenic subset of grafted mouse cells. In addition, it has been suggested that the tissue culture environment itself has a negative effect on subsequent survival *in vivo*, perhaps by making cells more immunogenic (reviewed, Smythe *et al.* 2000).

Even under optimised experimental conditions, the efficiency of MTT rarely exceeds parity i.e. the number of donor-derived myonuclei is approximately equivalent to the original number of grafted cells (reviewed, Partridge 2003). Muscle formation is mainly localised to the graft site, and is not augmented with time (Morgan *et al.* 1993). Before MTT could have clinical application, the problems associated with cell survival, proliferation, differentiation and dispersal would need to be addressed effectively (reviewed, Smythe *et al.* 2001, Partridge 2003).

1.55 Gene therapy of DMD

The most encompassing therapeutic solution to DMD would be to insert a normal copy of the *dystrophin* gene into the patient's muscle cells, and thereby restore sufficient protein expression to ameliorate pathology. At 2.4 Mb, *dystrophin* is the largest known mammalian gene, consisting of 79 exons which produce 14 kb of mature mRNA and generate a 427 kDa protein (Roberts *et al.* 1993).

Its exceptionally large size makes the *dystrophin* gene difficult to accommodate in most vector systems (reviewed, Wells & Wells 2002, Kapsa *et al.* 2003, Bogdanovich *et al.* 2004). However, in an early study it was shown that full-length human

dystrophin cDNA could be ligated into plasmid vectors and subsequently expressed in both cultured cells and mouse muscles, albeit at very low levels (Acsadi *et al.* 1991). More recently, the efficiency of expression in comparable experiments has been increased by use of hyaluronidase pre-treatment and electrotransfer (Gollins *et al.* 2003, Ferrer *et al.* 2004) or semisystemic delivery through limb blood vessels (Liang *et al.* 2004). In an alternative approach, it has been shown that deletion of parts of the rod domain of *dystrophin* can be used to create 'mini-dystrophin' genes that both retain some function and are small enough to ligate into viral vectors (Wang *et al.* 2000, Roberts *et al.* 2002, reviewed, Wells & Wells 2002). Intramuscular injection of full-length human dystrophin cDNA is currently being employed in a clinical trial of gene therapy for DMD, conducted at the Pitie-Salpetriere Hospital in Paris (Thioudellet *et al.* 2002, Romero *et al.* 2002).

Both *mdx* and DMD muscles contain small numbers of 'revertant' muscle fibres that express near-normal levels of dystrophin protein (Hoffman *et al.* 1990, Lu *et al.* 2000). In the *mdx*, revertant fibres are formed by alternative processing of mRNA transcripts to skip the expression of up to 30 exons and yield a smaller, but functional protein (Lu *et al.* 2000). This endogenous phenomenon has been recapitulated by the use of antisense oligoribonucleotides to modify processing of *dystrophin* pre-mRNA and induce expression of a functional, alternatively-spliced dystrophin protein (Mann *et al.* 2001). The use of block copolymers as transfection reagents has been shown to increase the efficiency of this approach to the extent that substantial structural and functional improvements can be measured in treated *mdx* muscles (Lu *et al.* 2003).

1.6 Myotoxic agents

1.61 Use of myotoxic agents in the study of muscle regeneration

The progressive clinical decline of DMD patients is in part attributable to the failure of effective muscle regeneration (Dubowitz 1985, Dubowitz 1992, Rafael *et al.* 1997, Decary *et al.* 2000), a characteristic of human myopathies which is not adequately modelled by small animal homologues of muscular dystrophy such as the *mdx* mouse (Dangain & Vrbova 1984, Tanabe *et al.* 1986, Cullen & Jaros 1988, reviewed, Partridge 1997). Myotoxic agents that cause severe damage muscle tissue have been employed to generate better models of regeneration in adult rodent muscles.

Commonly used myotoxic preparations include the local anaesthetic bupivacaine, aqueous solutions of barium chloride, and snake venoms such as notexin and cardiotoxin (reviewed, Harris 2003). Although structurally diverse, all of these agents appear to elicit toxicity by interfering with cellular ion transportation, in particular of calcium.

1.62 Notexin

Notexin is a toxic phospholipase A₂ purified from the venom of the Australian Tiger snake *Notechis scutatus scutatus* (Halpert & Eaker 1976, Dixon & Harris 1996, reviewed, Harris 2003). When injected into skeletal muscle tissue, notexin binds to the sarcolemmas of slow (oxidative) myofibres where its hydrolytic activity induces the formation of small lesions. As a result of the consequent loss of ion gradients, parts of the myofibres hypercontract, the weakened intervening areas rupture and complete degeneration occurs (Dixon & Harris 1996, Harris *et al.* 2003, reviewed, Harris 2003). The cytoplasmic protein desmin is rapidly degraded with an estimated half-life of 1 h, whilst other structural proteins such as dystrophin and the contractile proteins actin and myosin have estimated half-lives of up to 9 h as determined by Western blotting or Coomassie blue staining of protein gels (Harris *et al.* 2003). By 6 h after injection, serum creatine kinase activity has increased by 5 or 6-fold, and remains elevated for more than 24 h (Pluskal *et al.* 1978). The muscle microcirculation and the basal laminae of myofibres remain intact (Harris & Cullen 1990).

It has been reported that fast glycolytic muscle fibres are relatively resistant to the effects of notexin (Harris *et al.* 1975, reviewed, Harris 2003). Nevertheless, notexin has been shown to bind to the sarcolemmas of fast fibres (Dixon & Harris 1996), and several studies have successfully used notexin to induce degeneration and regeneration in mouse TA muscles, which have a predominantly fast fibre-type profile (Lefaucheur & Sebillé 1995, Gross & Morgan 1999, Heslop *et al.* 2000).

Proliferating myoblasts rapidly accumulate around notexin-induced lesions (Klein-Ogus & Harris 1983, Dux *et al.* 1993, reviewed, Harris 2003), implicating them as the source of subsequent regeneration. This observation has been cited as evidence that satellite cells are insensitive to the effects of notexin (reviewed, Harris 2003).

1.63 Cardiotoxin

Cardiotoxins are polypeptides of 60-65 amino acids long which are derived from the venoms of cobras, and are general cytotoxins related to acetyl choline receptor-binding α -neurotoxins. Cardiotoxin is a pore-forming agent that causes depolarisation and degradation of the sarcolemmas of myofibres (Ownby *et al.* 1993, reviewed, Harris 2003). The basal lamina remains intact and acts as a scaffold for subsequent regeneration within the intact tube. 24 h after injection, mitochondria surviving within the tube are visibly swollen (Ownby *et al.* 1993). It is thought that failure of mitochondrial respiration, resulting from inactivation of calcium-dependant proteases and subsequent Ca^{2+} overload, is a major factor in the final stages of degeneration (reviewed, Harris 2003). This hypothesis is supported by the finding of an *in vitro* study which showed that cardiotoxin caused depolarisation of chick skeletal muscle cells, and that this effect was reduced by the addition of Ca^{2+} ions (Chen & Harvey 1993). Data from another *in vitro* study, using human and equine skeletal muscle cell cultures, showed that cardiotoxin causes breakdown of phospholipids and production of free fatty acids. This suggests that activation of endogenous phospholipase C activity may be involved in the degenerative process (Fletcher *et al.* 1991).

1.64 Bupivacaine

Bupivacaine ('*Marcaine*') is a local anaesthetic which causes necrosis and apoptosis of muscle fibres, but is thought to be non-toxic to other tissue components (Foster & Carlson 1980, Zink *et al.* 2003). Degeneration of myofibres and neuromuscular junctions occurs with 4 h of injection. Whilst the myofibres themselves regenerate effectively, significant ultrastructural changes to the neuromuscular junctions persist for several weeks (Nishizawa *et al.* 2003). Bupivacaine has been shown to both induce Ca^{2+} release from the sarcoplasmic reticulum and inhibit its uptake (Komai & Lokuta 1999, Zink *et al.* 2002). In addition, the contractile proteins become sensitised to Ca^{2+} (Zink *et al.* 2002). Increased cytosolic concentration of Ca^{2+} occurs concurrently with the release of cytochrome C and mitochondrial depolarisation (Irwin *et al.* 2002). The volume density of mitochondria declines, reaching its lowest value 24 h after injection (Nishizawa *et al.* 2003). Whilst intact fibres from the oxidative flexor digitorum brevis (FDB) and soleus muscles are sensitive to bupivacaine, fibres from the glycolytic oesophageal and EDL muscles are relatively

resistant to its activity. These findings suggest that oxidative metabolism is a major factor in bupivacaine toxicity, mediated through mitochondria (Irwin *et al.* 2002).

In tissue culture, bupivacaine is rapidly toxic to rat myotubes and inhibits myoblast fusion, although differentiation resumes normally on removal of the drug. This implies that it is relatively non-toxic to undifferentiated MPC (Schultz & Lipton 1978, Stygall *et al.* 1979).

1.65 Barium chloride

Injection of aqueous barium chloride solution into skeletal muscle causes necrosis of muscle fibres (Caldwell *et al.* 1990, Wells *et al.* 1998), but does not appear to affect the basement membrane, which acts as a scaffold for subsequent regeneration (Caldwell *et al.* 1990). The myonecrosis caused by barium chloride has been used to increase the efficiency with which plasmid (Wells *et al.* 1998) and retroviral (Fassati *et al.* 1995, 1996) DNA can be introduced into muscle fibres, and to augment muscle formation by human myoblasts grafted into mouse muscles (Pye *et al.* 2004).

1.7 Aims of study

The ability of satellite cells to contribute to differentiated myofibres is beyond doubt both *in vivo* (Moss & Leblond 1971) and *in vitro* (Rosenblatt *et al.* 1995). However, since other phenotypes also contribute to myonuclei (Ferrari *et al.* 1998, Seale *et al.* 2002, Camargo *et al.* 2003, Polesskaya *et al.* 2003) the precise role of satellite cells remains incompletely defined. Importantly, the mechanism by which the adult satellite cell compartment is renewed is not well understood (**Fig. 1.2**), and is fundamental to determining whether satellite cells function as adult stem cells, or are a population of intermediate progenitors that are the progeny of a separate stem cell population.

Current understanding of adult muscle regeneration is largely based on studies of whole muscles, in which the precise cellular source of regeneration is difficult to pinpoint, and on *in vitro* and *in vivo* studies of cell isolates, in which artefacts may be generated by the tissue culture environment itself, and moreover where satellite cells are removed from their physiological niche beneath the basal lamina and are difficult to distinguish from other cell types originally resident within the tissue. In both approaches it is difficult to formally correlate functional data with unequivocally identifiable *in vivo* phenotypes. All available data describing satellite cell behaviour are derived either from tissue culture experiments, studies of whole muscles, or transplantation studies of myoblasts which are removed from their endogenous niche, or perhaps never were within the satellite cell niche. A formal investigation of the potential of satellite cells has therefore been lacking.

Since satellite cells are defined by their anatomical position beneath the basal lamina of myofibres, they can only be isolated within preparations of intact single myofibres (Rosenblatt *et al.* 1995). Previously, studies of satellite cells in such preparations have been carried out *in vitro* (Rosenblatt *et al.* 1995, Zammit *et al.* 2002, 2004a). In the present study, single myofibres have been grafted into mouse muscles to investigate the behaviour of defined and discrete populations of satellite cells *in vivo*.

Overall aim:

This work has aimed to analyse the stem cell potential of satellite cells by formally investigating their contribution to skeletal muscle regeneration.

Specific aims:

- 1) To investigate the ability of the discrete population of satellite cells associated with a single intact myofibre to contribute to muscle after grafting, and to compare this ability with that of myogenic cells not maintained in association with myofibres.**
- 2) To compare the myogenic potential of satellite cells associated with single myofibres derived from three different hindlimb muscles: the EDL, soleus and TA.**
- 3) To investigate the ability of satellite cells to undergo a physiological process of self-renewal.**
- 4) To investigate the response of defined satellite cells to muscle tissue damage.**

Chapter 2

Materials and Methods

2.1 Mouse strains

All animals were bred and housed in the Biological Services Unit, Clinical Sciences Centre, Hammersmith Hospital, Imperial College Faculty of Medicine unless otherwise stated. Animal breeding and procedures were carried out in accordance with the Animals (Scientific Procedures) Act of 1986.

2.11 C57Bl/10ScSn (C57Bl/10) mouse

Mice of the inbred strain C57Bl/10ScSn were obtained from Harlan. This particular wild-type strain was selected because it forms the background of the *mdx* mutant strain.

2.12 C57Bl/10ScSn-*mdx* *nu/nu* (*mdx*-nude) mouse

The *mdx* mouse is a genetic and biochemical homologue of DMD that spontaneously arose within a C57Bl/10ScSn colony (Bulfield *et al.* 1984). The *mdx* mutation in the *dystrophin* gene results in the near-absence of dystrophin protein (Partridge 1997). Host *mdx* mice were bred onto a nude background (Partridge *et al.* 1989). Nude mice fail to develop a mature immune system as a result of absent thymus glands and are therefore immunotolerant of grafted cells.

2.13 3F-*nLacZ*-2E transgenic mouse

The 3F-*nLacZ*-2E transgenic mouse contains seven copies of a construct consisting of 2 kb upstream of the myosin light chain (MLC)-3F transcriptional start site, *nLacZ*-SV40 poly(A) in frame in the second MLC3F-specific exon, 1 kb of MLC3F sequence 3' of *nLacZ*, and a 260-bp 3' MLC1F/3F enhancer (Kelly *et al.* 1995). β -gal expression faithfully recapitulates fast myosin expression in the nuclei of all fast muscle fibres,

and is absent in the nuclei of slow muscle fibres and satellite cells (Kelly *et al.* 1995, Beauchamp *et al.* 2000).

2.14 *Myf5*^{*nlacZ/+*} mouse

The *Myf5*^{*nlacZ/+*} mouse has *nlacZ*-SV40poly(A) *RNApolIII/Neo* targeted to the first exon of the *Myf5* gene such that β -gal is produced as a fusion protein with the first 13 amino acids of *Myf5*. There is also a small deletion in the *Myf5* gene. *Myf5*^{*nlacZ/nlacZ*} homozygotes have early neonatal mortality as a consequence of abnormal rib development impairing respiratory effort (Tajbakhsh *et al.* 1996a). Heterozygous *Myf5*^{*nlacZ/+*} mice are viable. *Myf5*- β -gal fusion protein is expressed by both myonuclei and satellite cells during the perinatal period. In adults, *Myf5*- β -gal protein is expressed in the majority of satellite cell nuclei and also in the myonuclei of recently-regenerated muscle fibres (Tajbakhsh *et al.* 1996a, Beauchamp *et al.* 2000). *Myf5*^{*nlacZ/+*} mice were distinguished from negative littermates by identification of β -gal expression in the muscle of tail tip amputations.

2.15 *Pax3*^{*GFP/+*} mouse

In the *Pax3*^{*GFP/+*} mouse, a gene encoding green fluorescent protein (GFP) replaces the first exon of the *Pax3* gene in one allele, with the other allele encoding functional *Pax3* protein. The original gene-targeted line is a conditional knock-in. *Pax3*^{*GFP/+*} viable heterozygotes are generated by crossing the gene-targeted line with a flip mouse. Cytoplasmic GFP expression recapitulates expression of the *Pax3* gene (personal communication, F. Relaix). Cells derived from the *Pax3*^{*GFP/+*} mouse were obtained from Dr. D. Montarass of the Pasteur Institute, Paris.

2.16 *H-2K^b*-tsA58 mouse

H-2K^b-tsA58 transgenic mice express the tsA58 thermolabile mutant of the SV40 large T antigen, an immortalizing gene, under the control of the inducible *H-2K^b* promoter (Jat *et al.* 1991). Thermolabile tsA58 protein is expressed by cell isolates cultured in the presence of interferon- γ (IFN- γ) and is functional at 33°C. Cells cultured under these 'permissive' conditions thus become conditionally immortalized. Cell lines can be readily derived from the skeletal muscle tissue of both homozygote and heterozygote *H-2K^b*-tsA58 mice (Jat *et al.* 1991, Morgan *et al.* 1994).

Homozygotes were paired with C57Bl/10 and *Myf5^{nlacZ/+}* mice to obtain double heterozygotes, facilitating the generation of strain-specific satellite cell-derived lines.

2.2 Muscle cell culture protocols

All media were obtained as tissue culture grade. All procedures were carried out aseptically and excepting dissections, under a laminar flow hood. Sera and collagenase Type 1 were batch tested for myoblast and single myofibre culture.

2.21 Isolation and culture of single myofibres

Single myofibres were prepared from the EDL, TA and soleus muscles of mice according to the method described by Rosenblatt *et al.* (1995), with minor modifications.

(a) Media

All media were prepared on the day of use.

Collagenase solution: Collagenase Type 1 (Sigma) was freshly prepared at 2 mg/ml in Dulbecco's modified Eagle's medium (containing glucose 4500 mg/l) (DMEM) (Gibco) and sterilized by passing through a 0.2 µm filter (Sartorius).

Serum rinse: Serum rinse was prepared from DMEM supplemented with 10% (v/v) horse serum (HS) (Gibco) and passed through a 0.2 µm filter.

Serum-free medium: Serum-free medium was prepared from DMEM supplemented with 4 mM L-glutamine (L-glu) (Sigma) and 1% (v/v) penicillin and streptomycin solution (pen/strep) (Sigma).

Plating medium: Plating medium was prepared from DMEM supplemented with 4 mM L-glu, 1% (v/v) pen/strep, 10% (v/v) HS and 0.5% (v/v) chick embryo extract (CEE) (PAA Laboratories), passed through a 0.2 µm filter to remove debris, and warmed to 37 °C before use.

(b) Isolation of viable single myofibres from mouse muscles

Mice were killed by cervical dislocation. Hindlimbs were finely shaved using a scalpel blade, rinsed with 70% ethanol, pinned out on a dissecting board and the skin removed. Using curved Vanna scissors and fine forceps, relevant muscles were dissected intact (**Fig. 2.1**) by handling the tendons only and avoiding all application of tension. Dissected muscles were immediately placed in separate bijoux containing 2 ml of collagenase solution. Muscles were incubated at 35 °C in a shaking water bath for 1 h- 1 h 30 (EDL or soleus muscles) or 2-3 h (TA muscles), until myofibres were visibly starting to detach.

50 mm x 18 mm non-tissue culture Petri dishes (Sterilin) (to which cells are non-adherent) were rinsed with filtered HS and 8 ml of serum-free medium was added and incubated at 37 °C/ 5% CO₂ for at least 30 min prior to use. Muscles were transferred into prepared dishes after digestion. Single myofibres were liberated by trituration of muscles with a customized wide-mouthed, heat-polished Pasteur pipette that had been flushed with serum rinse to prevent cell adherence. Using a finer Pasteur pipette, groups of ~30 single myofibres were serially passed through 2-4 further dishes to remove debris and contaminating cell types. Only intact myofibres (those that were long, straight and without areas of hypercontraction or adherent debris) were used; all damaged myofibres were immediately discarded. Prepared myofibres were incubated at 37 °C/ 5% CO₂ before culture, fixation or grafting.

(c) Maintenance of single myofibres as adherent cultures

Single myofibres were maintained as adherent cultures in Primaria[®] 6-well and 24-well plates (Beckton Dickinson) (for optimal primary cell adherence) and 8-well Lab-Tek[®] chamber slides (Nalge Nunc International) which had been previously coated with the basement membrane matrix Matrigel (BD Biosciences) (1 mg/ml in DMEM) for 45 min at 37 °C. After removal of excess substrate, single myofibres were gently placed in separate wells (24-well plates or chamber slides) or evenly spaced at 6-10 myofibres/ well (6-well plates). Plating medium (250 µl for chamber slides, 0.5 ml for 24-well plates, 1.5 ml for 6-well plates) was added down the side of each well to avoid disturbing the myofibre, and cultures immediately transferred to an incubator maintained at 37 °C/ 5% CO₂.

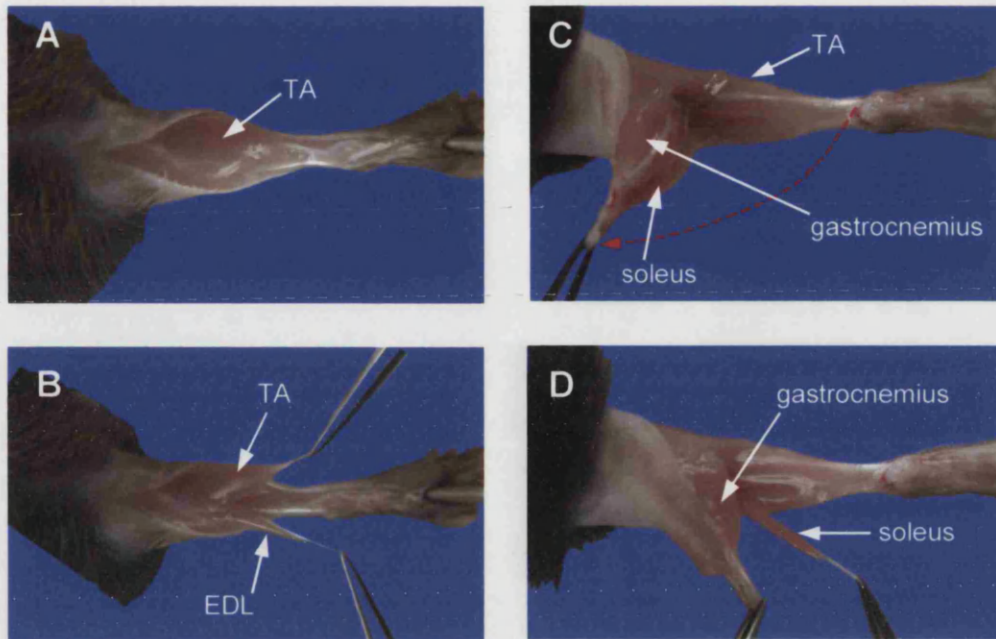


Fig. 2.1. Anatomical location of EDL, TA and soleus muscles in the mouse hindlimb. Partial dissection of EDL and TA muscles from the right hindlimb (A-B) and soleus muscle from the contralateral left hindlimb (C-D).

2.22 Isolation and culture of primary myoblasts

This protocol was developed to obtain cell suspensions from adult mouse TA, soleus and EDL muscles. Depending on the muscle of origin, $2-4 \times 10^4$ cells were typically obtained per mg of tissue of which 35-57% were myoblasts as defined by immunocytochemical detection of the intermediate filament protein, desmin (2.74), an early marker of the myogenic lineage (Li *et al.* 1994).

(a) Media

Growth medium: Growth medium was prepared from DMEM supplemented with 4 mM L-glu, 1% (v/v) pen/strep solution, 20% (v/v) heat-inactivated foetal calf serum (HI-FCS) (PAA Laboratories) and 2% (v/v) CEE. HI-FCS was prepared by incubating foetal calf serum at 60 °C for 30 min (to remove complement), and passing through a 0.2 µm filter before use.

Collagenase Type 1 solution: prepared as previously described (2.21).

Trypsin solution: Trypsin 10X stock solution (25 mg/ml porcine trypsin) (Sigma) was prepared at 2.5 mg/ml in Hanks' Balanced Salts solution without calcium and magnesium (HBSS) (Gibco) adjusted to 25 mM HEPES using 1M HEPES buffer (Sigma).

(b) Preparation of cell suspension

Muscles were dissected free of connective tissue, weighed and transferred to the laboratory in serum-free medium (2.22a). Muscle tissue was finely minced using scissors and digested in 5 ml collagenase Type 1 solution for 10 min at 35 °C in a shaking water bath. Minced muscle was triturated for 1 min using a wide-mouthed Pasteur pipette (pre-rinsed with growth medium) and the tissue allowed to re-settle. The resultant cell suspension, which in this 1st fraction is mainly non-myogenic cell types, was aspirated and discarded. The remaining tissue was digested in 5 ml trypsin solution for 10 min at 37 °C, triturated for 1 min, and the resultant 2nd cell suspension decanted into 5 ml growth medium to inhibit further enzymatic digestion. After a second round of trypsin digestion, the resultant 2nd and 3rd cell fractions were pooled and passed through a 40 µm cell sieve to remove debris. Cells were pelleted by

centrifugation for 10 min at 350 RCF and re-suspended in growth medium. A viable cell count (c) was performed before either culture (d) or cryopreservation (2.24).

(c) Viable cell count

100 μ l Trypan blue dye (0.5% w/v in PBS) was added to a 200 μ l aliquot of cell suspension. Dye-excluding cells were counted in the marked area of a Mod-Fuchs Rosenthal haemocytometer chamber (BDH) (depth 0.2 mm) and the number of viable cells in the original sample calculated.

(d) Culture of primary myoblasts

Primary mouse myoblasts were seeded in growth medium at 10^3 cells/ cm^2 in tissue culture flasks (25 cm^2 , 75 cm^2 or 175 cm^2) (BD Falcon) coated with Matrigel (0.1 mg/ml in DMEM) as previously (2.21c). Culture at this relatively low density prevents terminal differentiation. Flasks were incubated at 37 °C/ 5% CO_2 . Growth medium was replaced every 48-72 h.

Cultures were passaged at 30-40% confluence ($\sim 10^4$ / cm^2). Growth medium was removed and cells briefly washed with phosphate buffered saline (PBS). Cells were detached by incubation with trypsin-EDTA solution (~ 20 μ l/ cm^2) (containing 0.5 g porcine trypsin and 0.2 g EDTA/ l) (Sigma) for 3-5 min at 37 °C. Detached cells were resuspended in at least three times the volume of growth medium, pelleted by centrifugation, resuspended in growth medium and a viable cell count performed (c) before re-seeding in fresh flasks.

2.23 Culture of conditionally immortal myoblast cell lines

(a) Derivation of satellite cell-derived cell lines

Conditionally immortal satellite cell-derived cell lines were obtained from *H-2K^b-tsA58/C57Bl/10* and *H-2K^b-tsA58/Myf5^{nLacZ/+}* double heterozygote mice (2.16). Single myofibres were isolated and cultured for 48 h in 24-well plates as previously described (2.21) by which time numerous satellite cells had migrated and started to proliferate. Myofibres were then removed, and cultures transferred to permissive conditions (b).

(b) Culture under conditions permissive for tsA58 expression

Cells were maintained as previously described (2.22) except that growth medium was supplemented with interferon- γ (IFN- γ) (Life Technologies) (20 U/ml) and cultures incubated at 33 °C/ 10% CO₂. Under these conditions, *H-2K^b*-tsA58-derived myoblasts undergo immortalization (2.16), proliferate rapidly (Morgan *et al.* 1994) and can be maintained for 40+ passages without losing the ability to differentiate.

(c) Culture under conditions non-permissive for tsA58 expression

Cultures were maintained in the absence of IFN- γ and at 37 °C/ 5% CO₂, as previously described for primary myoblasts (2.22). Under these conditions, functional tsA58 protein is not expressed (2.16) and immortalized myoblasts cease to proliferate after ~1 further population doubling, but retain the ability to differentiate (Morgan *et al.* 1994).

(d) Cloning by dilution method

This method was used to obtain single cell-derived lines. Myoblasts were seeded at an average density of 1 cell/ well in 96-well plates (Falcon) and cultured under permissive conditions as previously described (a). After 6 h, wells containing single cells were marked. After ~6 d in culture, resultant clonal cultures were passaged (2.22) and expanded.

2.24 Induction of differentiation

(a) Medium

Fusion medium: Fusion medium was prepared from DMEM supplemented with 4 mM L-glu, 1% pen/strep solution (v/v) and 5% HS (v/v).

(b) Culture conditions for differentiation

Cells were seeded at 2×10^4 / cm² in flasks or chamber slides coated with Matrigel (0.1 mg/ml in DMEM) as previously described (2.21). Cells were cultured at 37 °C/ 5% CO₂ for 24 h in growth medium (2.22), during which time they had approximately doubled in confluence and started to align and elongate. Growth medium was replaced with fusion medium and cultures incubated for a further 72 h, by which time

myoblasts had differentiated and fused to form multinucleate contractile myotubes. Cells in primary single myofibre cultures were induced to differentiate after reaching an approximately similar density by replacing plating medium with fusion medium and culturing for a further 72 h.

2.25 Cryopreservation of cells

(a) Medium

Freezing medium: Freezing medium was prepared from growth medium (2.23a) supplemented with 10% (v/v) dimethyl sulphoxide (Sigma) and sterilized by passing through a 0.2 µm filter.

(b) Storage of cells under liquid nitrogen

Cells were suspended at 10^5 - 10^6 / ml in freezing medium and aliquoted into 1 ml cryovials (Nalgene). Cells were initially frozen to -80 °C in an isopropanol cell freezer, ensuring a steady temperature decline of 1 °C/ min. Cryovials were then placed under liquid nitrogen at -196 °C.

2.3 Viral infection of cell cultures

2.31 LNPOZ retroviral vector

The LNPOZ retroviral vector contains both *β-gal* and *neomycin resistance (neo)* genes driven by the viral long terminal repeat (LTR). It also contains a poliovirus 5' non-translated region (NTR) which acts as an internal ribosome entry site and thus promotes efficient expression of the *β-gal* and *neo* genes from polycistronic message (Adam *et al.* 1991).

Frozen stocks of LNPOZ retroviral supernatant produced from the high titre ecotrophic packaging cell line, AmpliGPE (Takahara *et al.* 1992), were obtained from Dr J.E. Morgan.

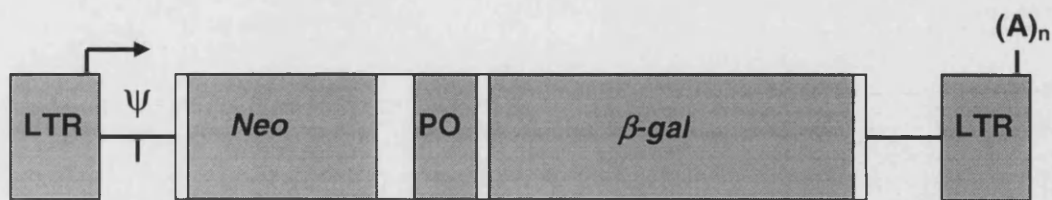


Fig. 2.2. The LNPOZ retroviral vector. LTR= long terminal repeat; ψ = viral encapsidation sequence (psi site); *neo* = *neomycin resistance* gene; PO = poliovirus 5' non-translated region (internal ribosomal entry site), β -gal = β -galactosidase reporter gene; $A_{(n)}$ = polyadenylation sequence.

2.32 Infection of satellite-derived cells with LNPOZ retroviral vector

(a) Media

Retroviral medium: Retroviral medium was prepared from LNPOZ retroviral supernatant diluted 1:5 (v/v) in growth medium (2.22). This titre infects up to 70% of cells in proliferating myoblast cultures, although the number of insertions per nucleus is not known (J. Morgan, personal communication). To aid the binding of viral particles to the surface of cells, Polybrene™ (hexadimethrine bromide, Sigma) was added at 8 μ g/ml. Medium was sterilized by passing through a 0.2 μ m filter. Retroviral supernatant was thawed and retroviral medium prepared immediately before use to prevent any loss of viral titre.

Antibiotic selection medium: Antibiotic selection medium was prepared from DMEM supplemented with 20% (v/v) HI-FCS, 2% (v/v) CEE, 4 mM L-glu and the neomycin analogue, G418 (Sigma) at 600 μ g/ml. Medium was sterilized by passing through 0.2 μ m filter before use. To determine the appropriate concentration of G418 for efficient selection, *H-2K^b*-tsA58 myoblasts were maintained in selection media containing different concentrations of G418 for 2 weeks. 600 μ g/ml was found to be the minimum concentration required to give 100% cell mortality at this timepoint (data not shown).

(b) Infection of cell cultures with LNPOZ retrovirus

Single myofibres were isolated from the EDL muscles of *H-2K^b-tsA58/C57Bl/10* mice (2.16) and maintained as adherent cultures as previously described (2.21). Single myofibres were removed after 2 d in culture, by which time satellite cells had migrated and started to proliferate. To retrovirally infect satellite cell-derived cells, plating medium was removed and cultures incubated with retroviral medium for 2 h at 37 °C/5% CO₂. After infection, retroviral medium was replaced with growth medium (2.22) and cells cultured under permissive conditions as previously described (2.23).

(c) Antibiotic selection and production of cell lines

After 72 h in culture post-infection, cells were passaged (2.22) and maintained under permissive conditions as previously described (2.23), except that antibiotic selection medium was used in place of growth medium. After 2 weeks, aliquots of cells were seeded in chamber slides, cultured for 48 h and fixed (2.71). X-gal staining (2.77) was used to confirm the presence of β -gal activity in the cytoplasm of infected cells. Immunocytochemistry for desmin protein (2.74) was used to confirm that infected cells were of the myogenic lineage (Li *et al.* 1994). Myogenic satellite cell-derived cell lines with cytoplasmic β -gal expression were produced by dilution cloning (2.23) of G418-selected cultures.

2.33 GFP lentiviral vector

Frozen stocks of concentrated GFP lentiviral vector (RRL.ppt.hPGK.GFP.pre.sin.-18) produced in the human embryonic kidney cell line, 293T, were obtained from Ms. K. Brimah. This vector expresses cytoplasmic-localising eGFP and is similar to that described by Piacibello *et al.* (2002), except that expression is under the control of the *hPGK* promoter. It is incorporated into human and mouse cells independent of cell cycle stage, and so efficiently infects both quiescent and post-mitotic phenotypes.

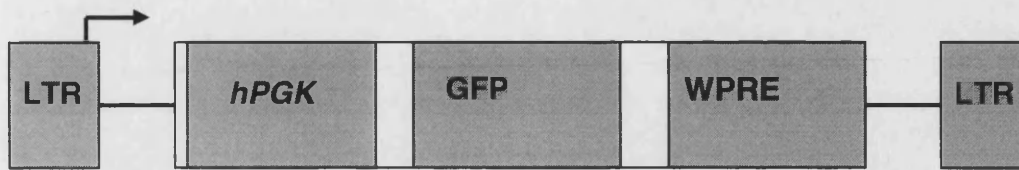


Fig. 2.3. GFP lentiviral vector. LTR= long terminal repeat, GFP= GFP reporter gene, hPGK= hPGK promoter, WPRE= mRNA-stabilising post-transcriptional regulatory element from woodchuck hepatitis virus.

2.34 Infection of isolated myofibres with GFP lentiviral vector

(a) Medium

Lentiviral medium

Lentiviral medium was prepared from lentiviral stock diluted 1:250 in plating medium (2.21). To aid the binding of viral particles to the surface of cells, Polybrene™ was added at 8 µg/ml. Medium was sterilized by passing through a 0.2 µm filter. Lentiviral stock was thawed and lentiviral medium prepared immediately before use to prevent any loss of viral titre.

(b) Infection of satellite cells with GFP lentivirus

An equal volume of freshly-prepared lentiviral medium was added to single myofibres suspended in plating medium (2.21), to give a final titre of 1:500 lentivirus. This titre has been found to label >99% of satellite cells in single myofibre preparations (personal communication, K. Brimah). Single myofibres were incubated at 37 °C/5% CO₂ for 2 h, and then removed from the medium and washed by serial transfer through four dishes of warmed serum-free medium (2.21).

2.4 Assays of cell behaviour

2.41 BrdU assay for DNA synthesis

Replicating cells in myoblast cultures were identified by assaying incorporation of BrdU (5-bromo-2'-deoxyuridine) into DNA precursors during mitosis, according to the protocol of Kaufman & Foster (1988). Conditionally-immortal myoblasts were seeded in chamber slides and cultured as previously described (2.23). Cultures were labeled overnight with 4 μ M BrdU (Sigma) added to growth medium, and then fixed with 95% ethanol (2.71). Fixed cultures were treated with 2 M HCl for 30 min at RT, and then neutralized with 3 x 20 min washes in 50 mM NaCl / 100 mM Tris HCl (pH 7.4). Myoblasts that had recently undergone DNA synthesis were identified by immunocytochemistry for BrdU (2.74), and their percentage of the total population calculated after counting ~100 cells under a light microscope.

2.42 Myoblast differentiation assay (fusion index)

Myoblasts were cultured in chamber slides under conditions optimized for differentiation as previously described (2.24). After 96 h, cultures were fixed with formalin (2.71) and nuclei stained with haematoxylin (2.78). Fusion index was calculated as the percentage of nuclei within myotubes (defined as cells with ≥ 3 nuclei).

2.5 Animal protocols

2.51 Host mice for cell grafting experiments

Graft recipients were *mdx*-nude mice (2.12) aged 24 d at the time of grafting, during the peak of dystrophic necrosis (Partridge 1997). When *mdx* muscle is grafted with wild-type muscle precursor cells, donor-derived muscle formation can be identified using immunohistochemistry for dystrophin protein (Partridge *et al.* 1989).

2.52 Anaesthesia and analgesia

(a) Anaesthesia

For irradiation (2.53), mice were lightly anaesthetised for humane restraint by subcutaneous injection of 50 µl of Hypnorm (79 µg/ml fentanyl citrate, 2.5 mg/ml fluanisone) (Janssen-Cilag Ltd) and Midazolam (1.25 mg/ml midazolam) (CP Pharmaceuticals Ltd) prepared in sterile water. For cell grafting (2.54, 2.55) and muscle damage (2.56) procedures, inhalable isoflurane (Schering-Plough Animal Health) was used to achieve stable anaesthesia. After all protocols, mice were maintained on a heated pad and given dampened food on the floor of the cage until full recovery.

(b) Analgesia

Mice used in muscle damage experiments (2.56) were given buprenorphine (0.05 mg/kg) (Vetergesic[®], Reckitt & Colman Products Ltd) analgesia by subcutaneous injection before recovery from anaesthesia.

2.53 Ablation of the satellite cell compartment by local irradiation

To ablate the satellite cell compartment of replication-competent satellite cells and create an optimized environment for donor-derived muscle formation, 3 d before grafting the hindlegs of anaesthetized (2.52) host mice were exposed to 18 Gy γ -irradiation delivered at 0.7 Gy/min from an IBL 637 irradiator (CIS Biointernational) (Gross *et al.* 1999). The bodies of mice were shielded by 4 cm lead.

2.54 Grafting of myoblasts into mouse muscles

Host mice were anaesthetized (2.52) and hindlegs held in position with tape. The skin of each hindleg was swabbed with 70% ethanol and a small incision made in the skin overlying the base of each TA muscle. 4 µl of viable (2.22) myoblasts, suspended at high known density in growth medium (2.22) were drawn into a 5 µl Hamilton syringe and inserted into the TA muscle under microscopic observation. Where necessary, the incision was closed with suture silk.

2.55 Grafting of single myofibres into mouse muscles

Fine needles were prepared from glass PCR pipettes drawn out in a hot flame. Freshly prepared single myofibres, suspended in 2-4 μ l serum-free medium (2.21) were drawn into needles and inserted into recipient muscles as previously described (2.54). The needle was flushed out under microscopic observation to confirm the absence of the myofibre.

2.56 Model of acute muscle damage

The myotoxic snake venom, notexin (reviewed, Harris 2003), was used to induce acute damage to engrafted muscles.

4 weeks after grafting (2.54, 2.55), host mice were anaesthetized (2.52) and 10 μ l purified notexin (Latoxan) (10 μ g/ml in PBS) injected into engrafted TA muscles using a 5 μ l Hamilton syringe (2 x 5 μ l injections into the same site). In some experiments, notexin was injected at the time of grafting; here only 5 μ l was used since the much smaller muscles of these younger mice were found to expel greater volumes.

2.6 Tissue preparation for histological analyses

2.61 Removal and storage of muscles

(a) Reagent

Gum tragacanth: Gum tragacanth (Sigma) (6% w/v) was slowly dissolved in sterile water for several hours at room temperature, and then heated overnight at 60 °C. Aliquots were stored at 4 °C until use.

(b) Removal of muscles for histology and immunohistochemistry

Mice were killed by cervical dislocation. Relevant muscles were dissected, bisected and mounted in gum tragacanth on cork discs. Muscles were snap frozen in isopentane cooled in liquid nitrogen and then stored at -80 °C.

2.62 Preparation of cryosections

A Leica CM 1800 cryostat maintained at -18 °C was used to obtain cryosections of frozen muscles. 3-4 7 µm sections were cut at 100 µm intervals and collected onto silane-coated glass slides. After air-drying for 45 minutes, slides were wrapped in foil and stored at -80 °C before staining.

2.7 Staining of cells and tissue sections

2.71 Fixation methods

(a) 4% paraformaldehyde

Aliquots of 4% paraformaldehyde (PAF) in PBS were prepared, stored at -20 °C and defrosted immediately before use. Cells and tissues were fixed for 5 min at RT and then washed for 3 x 10 min with PBS. For fixation of single myofibres, 4% PAF was warmed to 37 °C and added directly to the culture medium at a final concentration of 2-3%.

(b) Methanol/ acetone

Ice-cold methanol/ acetone (1:1) was used to fix cell cultures on ice for 10 min. For adherent myofibre cultures, fixative was added directly to the culture medium to a final concentration of ~90% (v/v). Fixed cultures were washed for 3 x 10 min with PBS.

(c) 95% ethanol

Cultures were fixed with ice-cold 95% ethanol for 10 min on ice, and then washed for 2 x 10 min with PBS.

(d) 0.5% gluteraldehyde

Cold 0.5% gluteraldehyde in PBS was used to fix cells and tissues on ice for 10 min, before rinsing and then washing for 10 min with 2mM MgCl₂ in PBS.

(e) Formalin

10% buffered formalin solution was used to fix tissues at room temperature for 10 min, before thorough washing in distilled water.

2.72 Primary antibodies for protein immunochemistry

Target protein	Clone	Species	Source/ ref.	Dilution	Fixation method
desmin	D33 (monoclonal)	mouse	Dako	1:50-1:100	methanol/acetone
dystrophin	P7 (polyclonal)	rabbit	Dr Q.L. Lu	1:500	none
Pax7	(monoclonal)	mouse	Hybridoma bank	1:50	4% PAF
MyoD	(monoclonal)	mouse	Dako	1:50	4% PAF
MyoD	sc-760 (polyclonal)	rabbit	Santa Cruz	1:50	4% PAF
myogenin	sc-576 (polyclonal)	rabbit	Santa Cruz	1:50	4% PAF
β-gal	(polyclonal)	rabbit	Molecular Probes	1:50	4% PAF
GFP	(polyclonal)	rabbit	Molecular Probes	1:100	4% PAF
SV40 T antigen	Ab-2 (monoclonal)	mouse	Oncogene	1:100	4% PAF
BrdU	M0744 (monoclonal)	mouse	Dako	1:20	95% ethanol
neonatal myosin	BF34 tissue culture supernatant (monoclonal)	mouse	Borrione <i>et al.</i> (1988)	1:15	none
slow myosin	NOQ7.5.4D (monoclonal)	mouse	Sigma	1:50	none
fast myosin	MY32 (monoclonal)	mouse	Sigma	1:40	none
laminin	(polyclonal)	rabbit	Sigma	1:50	4% PAF

Table 2.1. Primary antibodies.

2.73 Antibodies for secondary detection

Target	Species	Detection method	Source	Dilution	Blocking method
Mouse Ig	goat	AlexaFluor 488 (green)	Molecular Probes	1:200	20% goat serum
Mouse Ig	goat	AlexaFluor 594 (red)	Molecular Probes	1:200	20% goat serum
Rabbit Ig	goat	AlexaFluor 594 (red)	Molecular Probes	1:200	20% goat serum
Rabbit Ig	swine	TRITC (red)	Dako	1:100	10% swine serum and 10% goat serum
Rabbit Ig	goat	AlexaFluor 488 (green)	Molecular Probes	1:200	20% goat serum
Mouse Ig	goat	Horse radish peroxidase	Dako	1:200	5% HS

Table 2.2. Secondary antibodies for fluorescent and peroxidase detection of mouse and rabbit immunoglobulins (Ig).

2.74 Immunocytochemistry of cell cultures

Immunocytochemistry was performed directly on cells and adherent single myofibres cultured in chamber slides or 24-well plates, and on cells spotted onto silane-coated glass slides and air-dried. Cells were fixed (2.71), and to reduce background caused by non-specific antibody binding, incubated for 20 min with blocking solution prepared from the pertinent serum (2.73) in PBS. The primary antibody (2.72) was diluted in the same blocking solution and applied to cultures for 1 h at room temperature (RT), followed by washing for 3 x 10 min in PBS and a further 1 x 10 min in blocking solution. The secondary antibody (2.73) was diluted in blocking solution and applied for 45 min at RT, followed by washing for 4 x 10 min in PBS. For immunofluorescent detection, stained cultures were air-dried for 1 h at RT and then mounted with coverslips and Fluorescent mounting medium (Dako) containing

the fluorescent nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI) at 100 ng/ml. Peroxidase detection was performed using Sigmafast[®] 3,3'-diaminobenzidine and urea hydrogen peroxidase tablet sets (Sigma) dissolved in 5 ml PBS and applied to cells for 5 min at RT. Cells were rinsed 3 times with distilled water (dH₂O), air-dried and mounted with aqueous mounting medium (Faromount[®], Dako) and coverslips.

For detection of nuclear proteins, blocking was preceded by permabilisation of fixed cells with Triton X 100 (Sigma) (0.1% v/v in PBS) followed by washing in PBS for 3 x 10 min.

In some experiments, 2 primary antibodies raised in different species were applied together and differentially detected using immunofluorescence.

2.75 Immunocytochemistry of single myofibres

Immunocytochemistry of single myofibres in suspension was performed similarly to that of cell cultures (2.74), but with certain modifications. 10% FCS was added to the appropriate blocking solution (Table 2.2), and to antibody preparations. Primary antibodies were applied overnight at 4 °C. For detection of nuclear proteins, blocking was preceded by permeabilisation of fixed myofibres with Triton X 100 (0.5% v/v in PBS). Single myofibres were individually placed on silane-coated slides and air-dried for a few minutes before mounting (2.74).

2.76 Immunohistochemistry of muscle tissue sections

Slides were thawed and sections were ringed with a water-repellent pen. Immunohistochemistry performed on fixed (2.71) or unfixed cryosections (2.62) as described for cell cultures (2.74). Unfixed sections were rehydrated with PBS for 5 min at RT prior to staining.

2.77 X-gal staining for localisation of β -gal activity

This protocol was based on that described by Gross & Morgan (1999), with minor modifications.

(a) Reagents

Detergent solution: Detergent solution was prepared from PBS with 2 mM MgCl₂, 0.02% (v/v) IGEPAL CA-630 (Sigma) and 0.01% (w/v) sodium deoxycholate.

X-gal stock: X-gal stock was prepared from 5-bromo-4-chloro-3-indolyl-B-D-galactopyronoside (BDH) 40 mg/ml in dimethyl sulphoxide.

X-gal diluant: X-gal diluant was prepared from PBS with 2 mM MgCl₂, 0.02% (v/v) IGEPAL CA-630, 0.01% (w/v) sodium deoxycholate, 5 mM K₃Fe(CN)₆ and 5 mM K₄Fe(CN)₆.

(b) Protocol

Cell cultures and tissue sections were fixed with 0.5% glutaraldehyde (2.71) and incubated in detergent solution for 10 min on ice. Single myofibres in suspension were fixed with 4% PAF only (2.71). A solution of the chromogenic β -gal substrate, X-gal, was prepared by diluting X-gal stock to 1 mg/ml in X-gal diluant. Slides and single myofibres in suspension were incubated in X-gal solution overnight at 37 °C, rinsed 3 times with distilled water, briefly air-dried and mounted with aqueous mounting medium and coverslips. Single myofibres isolated from 3F-*nLacZ*-2E mice were incubated in X-gal solution for 2 h only. The bright blue reaction product of X-gal localises β -gal activity to the nuclei or cytoplasm of cells.

2.78 Haematoxylin and eosin staining

(a) Use of haematoxylin and eosin to stain animal tissues

Haematoxylin is a basic dye which stains acidic structures a purplish blue colour. It has an affinity for organelles with high DNA or RNA content, such as nuclei, ribosomes and rough endoplasmic reticulum. Eosin is an acidic dye which has affinity for basic proteins such as those abundant in the cytoplasm, staining them pinkish red. When animal tissues such as skeletal muscle are stained with haematoxylin and eosin (H & E), nuclei therefore appear blue and cytoplasm pink (Burkitt *et al.* 1993).

(b) Reagents

Haematoxylin: Gill's haematoxylin (containing ethylene glycol) (BDH) was used.

Eosin: Eosin stain was prepared from Eosin (1% w/v) (Sigma), Erythrosin B (0.4% w/v) and Phloxine B (0.2% w/v) (Sigma) in dH₂O, and passed through filter paper before use.

(c) Staining of tissue sections with H & E

Cryosections were thawed and fixed with formalin (2.71). Slides were stained with haematoxylin for 3 min, and then washed in tap water until it ran clear. Slides were stained with eosin for 1 min, and again washed in tap water. Dehydration was performed by incubating in 70% ethanol (30 secs), 90% ethanol (30 secs) and 100% ethanol (2 x 30 secs). Slides were then incubated in the histological clearing agent Histo-Clear™ (National Diagnostics) (2 x 30 secs). Slides were air-dried and mounted with DPX mounting medium (R.A. Lamb Lab. Supplies).

2.79 Microscopy and image capture

(a) Light microscopy

Brightfield microscopy of fixed cells and tissues was carried out using a Zeiss Axiophot microscope and Metamorph® software. Light microscopy and image capture of live cell cultures was carried out using an Axiovert 100 microscope and Sirius software.

(b) Fluorescence microscopy

Fluorescence microscopy and image capture was performed using a Zeiss Axiophot microscope and Metamorph® software.

Microsoft Photoshop 7 was used to make minor adjustments to some images.

Chapter 3

The myogenic potential of satellite cell grafts

3.1 Background and aim of study

3.11 Background to work

When grafted into the skeletal muscle of a new host, isolated myoblasts can participate in regeneration to form new differentiated muscle fibres (Watt *et al.* 1982, Partridge *et al.* 1989). However, the efficiency with which new tissue is formed is extremely low, rarely exceeding parity (reviewed, Partridge 2003), and does not reflect the latent regenerative capacity of the endogenous precursor population of skeletal muscles (Studitsky 1962, Carlson 1970, Heslop *et al.* 2000, Luz *et al.* 2002). This disparity suggests that although myoblast cell lines can be expanded extensively *in vitro*, most cells lose their proliferative competence when subsequently returned to an *in vivo* environment. The only cells of any lineage that have been demonstrated to maintain robust stem cell function after grafting are HSC, which by definition, can clonally repopulate the entire lymphohaematopoietic system of a lethally-irradiated mouse (Till & McCulloch 1961, Smith *et al.* 1991, Osawa *et al.* 1996).

Satellite cells are believed to be the main source of new myonuclei in regenerating muscles, and the ability of their progeny to contribute to differentiated muscle both *in vitro* (Rosenblatt *et al.* 1995) and *in vivo* (Moss & Leblond 1971) is unequivocal. Nevertheless, grafting experiments have failed to determine whether satellite cells are most appropriately defined as stem cells, with the ability to generate large numbers of differentiation-competent progenitors (Lajtha 1979, Moore 1979, Morrison *et al.* 1997, Anderson *et al.* 2001) or as intermediate progenitor cells, with a more modest proliferative capacity (Elwood 2004).

The majority of myogenic cells resident in muscle are thought to be radiation-sensitive (Wakeford *et al.* 1991, Quinlan *et al.* 1995), but a subset is resistant to the effects of radiation and can be recruited into an active myogenic program in response to acute damage (Heslop *et al.* 2000). Whilst this subset has been demonstrated to

give rise to new satellite cells (Heslop *et al.* 2000), it has not been established whether radiation-resistant cells themselves form a small subset of the satellite cell compartment, or whether they comprise a separate population.

There is increasing evidence that stem cell function is critically dependent on interaction with other differentiated cell types present in the tissue (Song *et al.* 2002b, Kaeffer 2002, Shen *et al.* 2004, Blanpain *et al.* 2004). Whilst the definitive anatomical position of satellite cells (Mauro 1961) involves intimate association with myonuclei, the functional significance of this relationship has been little investigated. The single myofibre isolation technique (Rosenblatt *et al.* 1995) allows defined populations of satellite cells to be isolated and studied within their endogenous niche beneath the basal lamina of intact myofibres. Carefully prepared single myofibre isolates (Rosenblatt *et al.* 1995) are extremely pure. When maintained as adherent cultures, only about 1 in 50 wild-type myofibres generate any desmin⁻ (non muscle) cells (Rosenblatt *et al.* 1995, Bockhold *et al.* 1998). Immunocytochemistry of isolated myofibres fails to identify associated cells expressing the endothelial marker Sca-1 (Asakura *et al.* 2002, Zammit & Beauchamp 2002), and microscopic examination shows that gross contaminants such as capillaries are not present. In contrast, preparations of single myofibres from *mdx* muscles frequently generate desmin⁻ cells (Bockhold *et al.* 1998) and sometimes have adherent capillaries. In preparations derived from non-dystrophic muscles, however, the single myofibre isolation technique is a reliable method for obtaining defined satellite cells that are both maintained in their endogenous niche, and are free from vascular or connective tissue contaminants.

Louise Heslop developed a novel technique in which isolated single myofibres were grafted into mouse muscles, allowing the myogenic potential of defined populations of satellite cells to be studied *in vivo*. Using grafts derived from EDL and soleus muscles, it was found that whilst most grafted myofibres apparently disappeared without trace, 13% of EDL myofibres and 34% of soleus myofibres were capable of giving rise to new muscle. Soleus myofibre grafts were found to generate between 8 and 200 new myofibres, whereas EDL myofibre grafts generated 101-300 new myofibres (L. Heslop, PhD thesis 2001). The small numbers of satellite cells originally present in the graft must necessarily have undergone very extensive

proliferation prior to differentiation into muscle. The findings of this preliminary study were cautiously interpreted as evidence for the existence of a rare, stem cell-like subset of satellite cells restricted to a small proportion of myofibres (T. Partridge, personal communication).

3.12 Aims of work

The objective of these experiments was to investigate the myogenic potential of satellite cells derived from different adult and juvenile muscles, and to generate quantitative data describing the relative myogenic potentials of grafts of single myofibres, conventionally-isolated primary myoblasts and a conditionally-immortalised satellite cell-derived myoblast cell line. An additional investigation aimed to determine the status of satellite cells as radiation-resistant or radiation-sensitive MPC.

3.13 Note on genetic markers of grafted cells

Most experiments described in this chapter use grafts derived from the 3F-*nLacZ*-2E mouse (2.13), in which β -gal activity reports fast myosin expression in the nuclei of fast muscle fibres (Kelly *et al.* 1995). In *mdx*-nude (2.12) muscles engrafted with 3F-*nLacZ*-2E cells, donor-derived muscle can be identified by the muscle-specific expression of two marker proteins, β -gal and dystrophin. The *nLacZ* transgene becomes silenced in long term cultures of myogenic cells (J. Morgan, personal communication). For this reason, in a myogenic cell line, retroviral infection with a *LacZ* gene was used as an alternative genetic labelling strategy.

3.2 Grafts of immortalised satellite cell-derived MPC

3.21 Aim

The objective of this study was to investigate the efficiency of muscle formation by grafts of different numbers of conditionally-immortalised MPC.

3.22 Preparation of a *LacZ* satellite cell-derived cell line

A cell line was generated from *H-2K^b*-tsA58 satellite cells retrovirally labelled with a cytoplasmic-localising *LacZ* gene. Single myofibres were isolated (2.21) from the EDL muscles of a 43-day-old male *H-2K^b*-tsA58/ C57 Bl/10 heterozygotic mouse (2.16), and cultured individually in the wells of 24-well plates (Fig. 3.1). After 24 h, examination of cultures by light microscopy showed that small numbers of satellite cells (0-4) had emanated from adherent myofibres. Myofibres were then removed. Cultures were infected with LNPOZ retrovirus (2.31, 2.32), and subsequently maintained under conditions permissive for expression of the tsA58 immortalising antigen (2.23), expanded, and passaged into flasks. The LNPOZ retrovirus expresses a *LacZ* gene, and a *neomycin resistance* gene that can be used as a selectable marker (2.31). Five single myofibre-derived cultures of infected cells (SF1-SF5) were selected for two weeks in medium containing the neomycin analogue, G418 (2.32). Two cultures, SF1 and SF3, yielded antibiotic-resistant cells. Aliquots of selected cells were seeded in chamber slides and either stained with X-gal to localise β -gal activity (2.77), or immunocytochemically stained for expression of desmin protein (2.74), a skeletal muscle lineage marker (Li *et al.* 1994). 100% of cells in SF1 and SF3 expressed desmin, suggesting that they were homogeneous cultures of skeletal myoblasts. 100% of cells in SF1 exhibited cytoplasmic β -gal expression, showing that the retrovirus had successfully integrated. None of the G418-resistant cells in SF3 expressed β -gal, suggesting that either (a) a clone had spontaneously developed G418 resistance in the absence of retroviral integration, or (b) that the retrovirus had integrated successfully but only the *neomycin resistance* gene, and not the *LacZ* gene, was expressed. Dilution cloning (2.23) was used to obtain 12 single cell-derived lines (SF1A-SF1K) from SF1 passage number (P) 11. X-gal staining (2.77) and desmin immunocytochemistry (2.74) of six of the expanded clones confirmed that 100% of cells expressed β -gal and desmin. Fusion assays (2.42) were used to compare the potential of the six clones to differentiate into myotubes *in vitro*; SF1K was found to

have the highest fusion index (63%) (**Fig. 3.2**). BrdU assays (**2.41**) showed that >99% of SF1K cells incorporated the label after culture under permissive conditions for 96 h, and >99% of cells did not incorporate label after culture under non-permissive conditions for 96 h. This demonstrated that the clone was conditionally immortalised, but unlikely to be transformed.

3.23 Muscle formation by different numbers of MPC

SF1K satellite-derived MPC were used to investigate the amounts of muscle formed by different numbers of grafted cells. The legs of 12 21-day-old *mdx*-nude host mice were exposed to 18 Gy γ -irradiation (**2.53**) to ablate the satellite cell compartment of replication-competent cells (Heslop *et al.* 2000), and create an optimised environment for donor-derived muscle formation (Gross *et al.* 1999). SF1K cells were expanded *in vitro*, and immediately before grafting, trypsinised, washed in growth medium, and a viable cell count performed (**2.22**). Cell suspensions of various densities were prepared. 3 d post-irradiation, host mice were anaesthetised (**2.52**) and cells grafted into the TA muscle of each hindleg (**2.54**). 5 legs were injected with growth medium only. 20 legs were grafted with 5×10^2 (n=5), 5×10^3 (n=4), 5×10^4 (n=4) or 5×10^5 (n=6) cells. After 3 weeks, engrafted muscles were removed, bisected, mounted and frozen in isopentane cooled in liquid nitrogen (**2.61**). Using a cryostat, two 7 μ l serial sections were taken at 100 μ m intervals throughout approximately 2/3 of the muscle body, and collected onto silane-coated glass slides (**2.62**). Donor-derived muscle was identified by localisation of β -gal activity in sections stained with X-gal (**2.77**). Numbers of donor-derived (β -gal expressing) myofibres (**Fig. 3.3**) were counted in representative mid-body sections.

There was a complete absence of β -gal expression in control muscles injected with growth medium only. Grafts of 5×10^2 cells formed 2.6 (± 5.3) myofibres; grafts of 5×10^3 cells formed 4.1 (± 3.1) myofibres; grafts of 5×10^4 cells formed 70.8 (± 111.1) myofibres, and grafts of 5×10^5 cells formed 271.6 (± 156.5) myofibres (**Fig. 3.4**). 3/5 grafts of 5×10^2 cells and 1/4 grafts of 5×10^3 cells formed no donor-derived muscle.

The Mann-Whitney test (see Appendix) was used to compare numbers of β -gal⁺ myofibres formed by the different numbers of grafted cells. There were significant

differences ($p < 0.05$) between the amount of muscle formed by grafts of 5×10^2 cells and grafts of 5×10^5 cells, and between the amount of muscle formed by grafts of 5×10^3 cells and grafts of 5×10^5 cells. Differences between other groups were not statistically significant (Mann-Whitney, $p > 0.05$). There was, however, a large difference between the mean amounts of muscle formed by grafts of 5×10^4 cells and grafts of 5×10^5 cells; the lack of statistical significance between these data sets is probably due to high standard deviations from the mean (SD).

Calculation of the grafting efficiency (the number of myofibres formed per grafted cell) showed that though grafts of larger numbers of cells formed greater numbers of donor-derived myofibres, grafts of smaller numbers of cells generated new muscle with greater relative efficiency (**Table 3.6**).

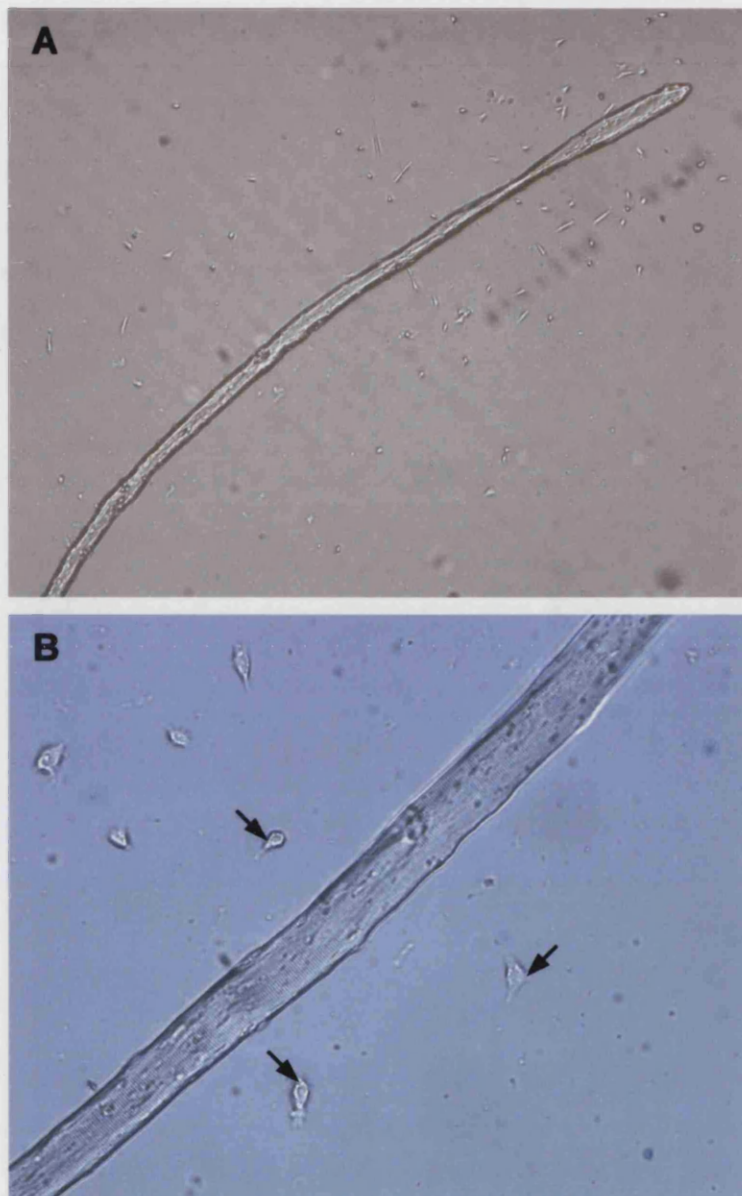


Fig. 3.1. Single myofibres in culture. Examples of satellite cell-derived myoblasts are arrowed. Original magnification x50 (A) or x200 (B).

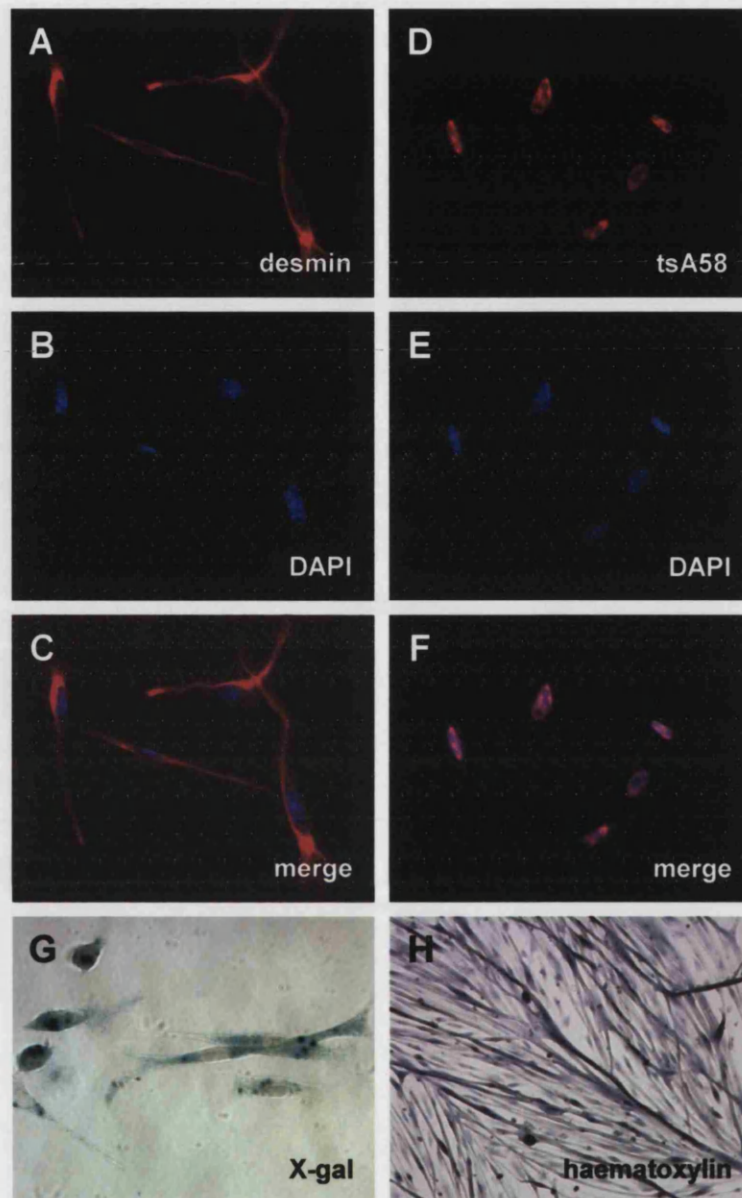


Fig. 3.2. Characteristics of SF1K conditionally immortalised satellite cell-derived clone. Fluorescence immunocytochemistry to detect expression of the myogenic lineage marker desmin (A-C) and under permissive conditions, the immortalising antigen tsA58 (D-F). X-gal staining to localise cytoplasmic β -gal activity (G). Differentiated myotubes stained with haematoxylin to calculate fusion index (H). Original magnification x400 (A-G) or x100 (H)

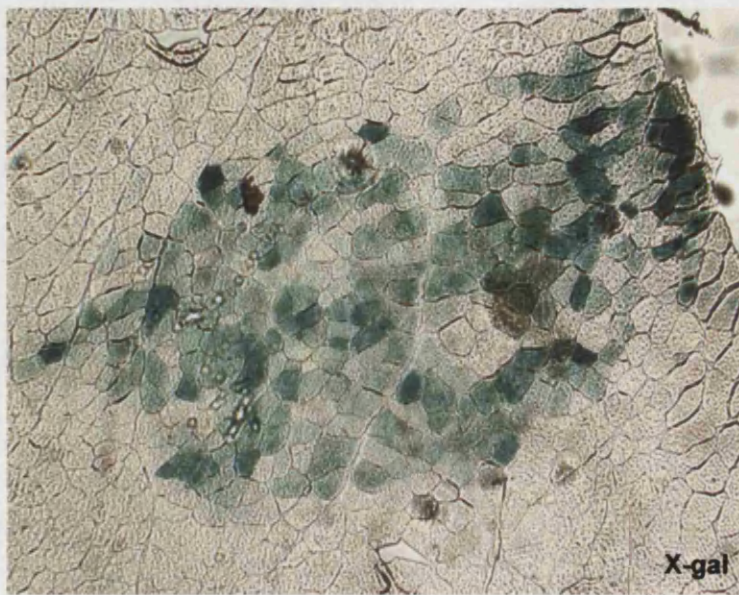


Fig. 3.3. Identification of donor-derived muscle in an irradiated *mdx*-nude TA muscle grafted with 5×10^5 clone SFIK conditionally-immortalised cells. After staining in X-gal solution, myofibres containing myonuclei derived from the graft are identifiable by cytoplasmic β -gal activity. Original magnification x100.

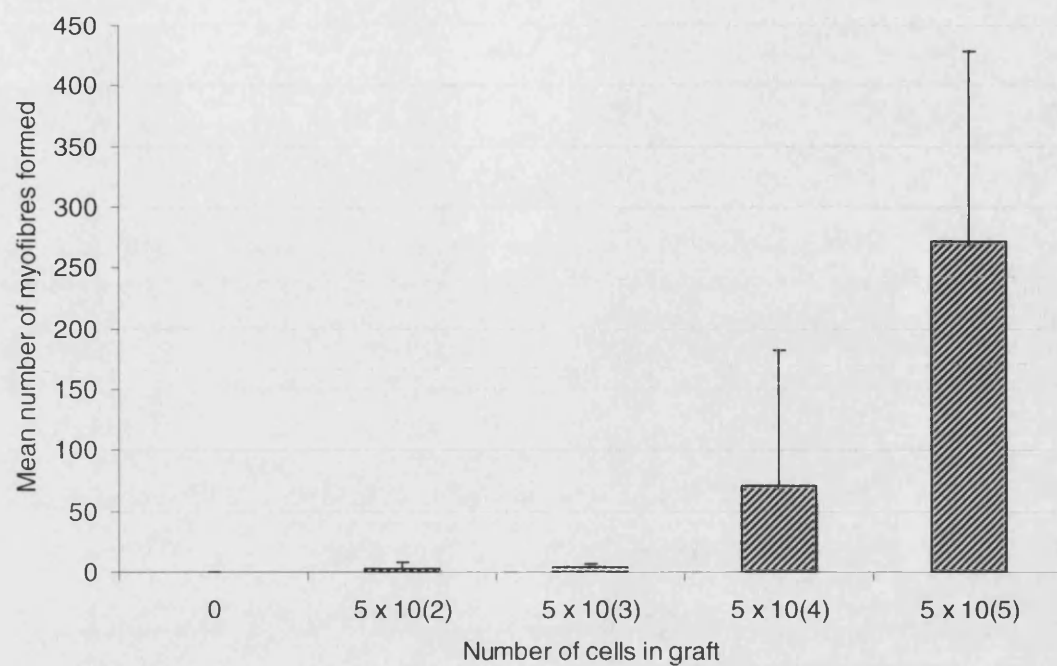


Fig. 3.4. Bar graph showing muscle formation by different numbers of grafted immortalised MPC. Different numbers of SFIK cells, which have cytoplasmic β -gal expression, were grafted into irradiated *mdx*-nude mouse muscles. After 3 weeks, the numbers of β -gal⁺ myofibres were counted in representative sections of engrafted muscles. These data show that the amount of muscle formed is proportional to the number of cells implanted, but not directly so. Error bars represent SD.

3.3 Grafts of primary MPC isolated from different muscles

3.31 Aim

The objective of this study was to investigate the comparative efficiency of muscle formation by primary MPC derived from three different hindlimb muscles: the EDL, soleus and TA.

3.32 Preparation of cell isolates from 3F-*nLacZ*-2E muscles

Cells isolates (2.22) were prepared from the muscles of ten 42-day-old 3F-*nLacZ*-2E mice (2.13) (five males and five females from two litters). TA, soleus and EDL muscles were dissected free of connective tissue and tendons, and muscle groups separately pooled. Muscles were minced and enzymatically disaggregated to obtain cell suspensions. >95% of isolated cells were viable as defined by exclusion of Trypan blue dye (2.22). 10 μ l of each cell suspension was spotted onto a silane-coated slide and air-dried at RT. The remaining cells were preserved in aliquots under liquid nitrogen. The percentage of myogenic cells in each isolate was determined by desmin immunocytochemistry of air-dried cells (2.74) (Fig. 3.5). The smooth muscle cells of the vasculature also express desmin protein, and may have made a minor contribution to counts. The number of myogenic cells obtained from different muscle groups is shown in Table 3.1.

	EDL	soleus	TA
Number of muscles	20	20	20
Mean weight (mg)	2.03	3.15	25.00
Viable cells per mg tissue	2.65×10^4	3.94×10^4	7.5×10^3
Desmin ⁺ cells (% of total)	36.85%	35.25%	53.70%
Desmin ⁺ cells per mg tissue	9.78×10^3	1.38×10^4	4.03×10^3
Mean desmin ⁺ cells per muscle	1.99×10^4	4.37×10^4	1.01×10^5

Table 3.1. Isolation of putative myogenic cells from the muscles of 42-day-old 3F-*nLacZ*-2E mice.

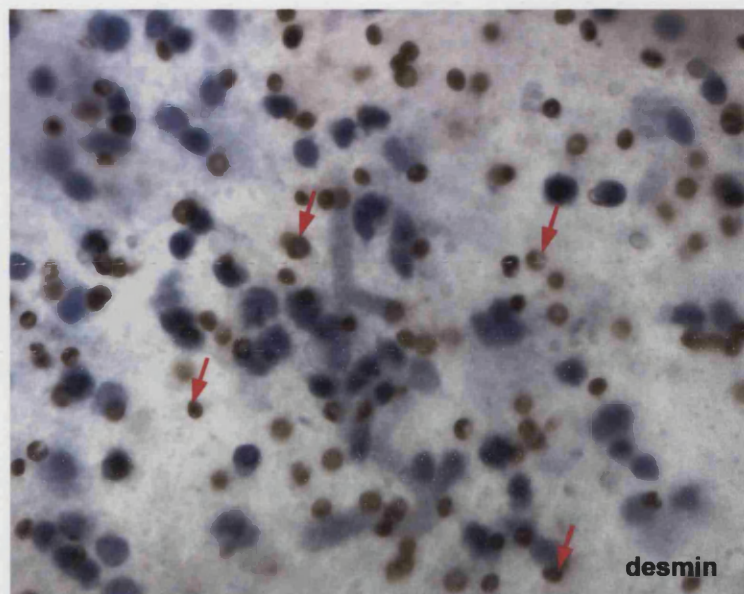


Fig. 3.5. Immunocytochemical detection of desmin protein in air-dried cells derived from enzymatic disaggregation of 3F-*nLacZ*-2E soleus muscles. Air-drying causes cells to lose their normal morphology but desmin protein remains detectable, in 35.25% of cells in this sample (arrows). Peroxidase secondary detection method, counterstained with haematoxylin. Original magnification x200.

3.33 The myogenic potential of MPC isolated from different muscles

This experiment aimed to compare the myogenic potential of enzymatically-disaggregated cells isolated from EDL, soleus and TA muscles. The legs of fourteen 21-day-old *mdx*-nude host mice were exposed to 18 Gy γ -irradiation three days prior to grafting (Gross *et al.* 1999) (2.53). On the day of grafting, cell isolates were thawed, washed in growth medium, and suspensions prepared such that 5×10^4 desmin⁺ cells were contained in 4 μ l growth medium. Therefore whilst the total number of grafted cells differed slightly between isolates derived from different muscles, the total number of putative myogenic cells within each graft was constant. Mice were anaesthetised (2.52) and 4 μ l cells (EDL isolates n=8; soleus isolates n=7; TA isolates n=8) or growth medium (n=5) was grafted into each TA muscle (2.54).

After 3 weeks, engrafted muscles were removed, bisected, mounted and frozen in isopentane cooled in liquid nitrogen (2.61). Using a cryostat, three serial 7 μ l sections were taken at 100 μ m intervals throughout approximately 2/3 of the muscle body, and collected onto silane-coated glass slides (2.62). In the first set of sections, nuclear β -gal activity was localised by staining in X-gal (2.77). In the second set of sections, expression of dystrophin protein was localised using fluorescence immunohistochemistry (2.76). For each engrafted muscle, the number of donor-derived myofibres was calculated by counting β -gal⁺ and dystrophin⁺ fibres in representative mid-body serial sections. In muscles injected with growth medium only, no β -gal activity was detected, but there were 2.4 (± 2.9) dystrophin⁺ revertant fibres. Muscles engrafted with cells each contained β -gal⁺ fibres that corresponded with dystrophin⁺ fibres in serial sections (Fig. 3.6).

Grafts of EDL cell isolates formed 110.8 (± 19.0) dystrophin⁺ myofibres of which 69.0 (± 27.9) were β -gal⁺; grafts of soleus cell isolates formed 110.7 (± 70.9) dystrophin⁺ myofibres of which 68.6 (± 65.3) were β -gal⁺; and grafts of TA cell isolates formed 122.6 (± 138.1) dystrophin⁺ myofibres of which 62.4 (± 39.4) were β -gal⁺ (Fig. 3.7).

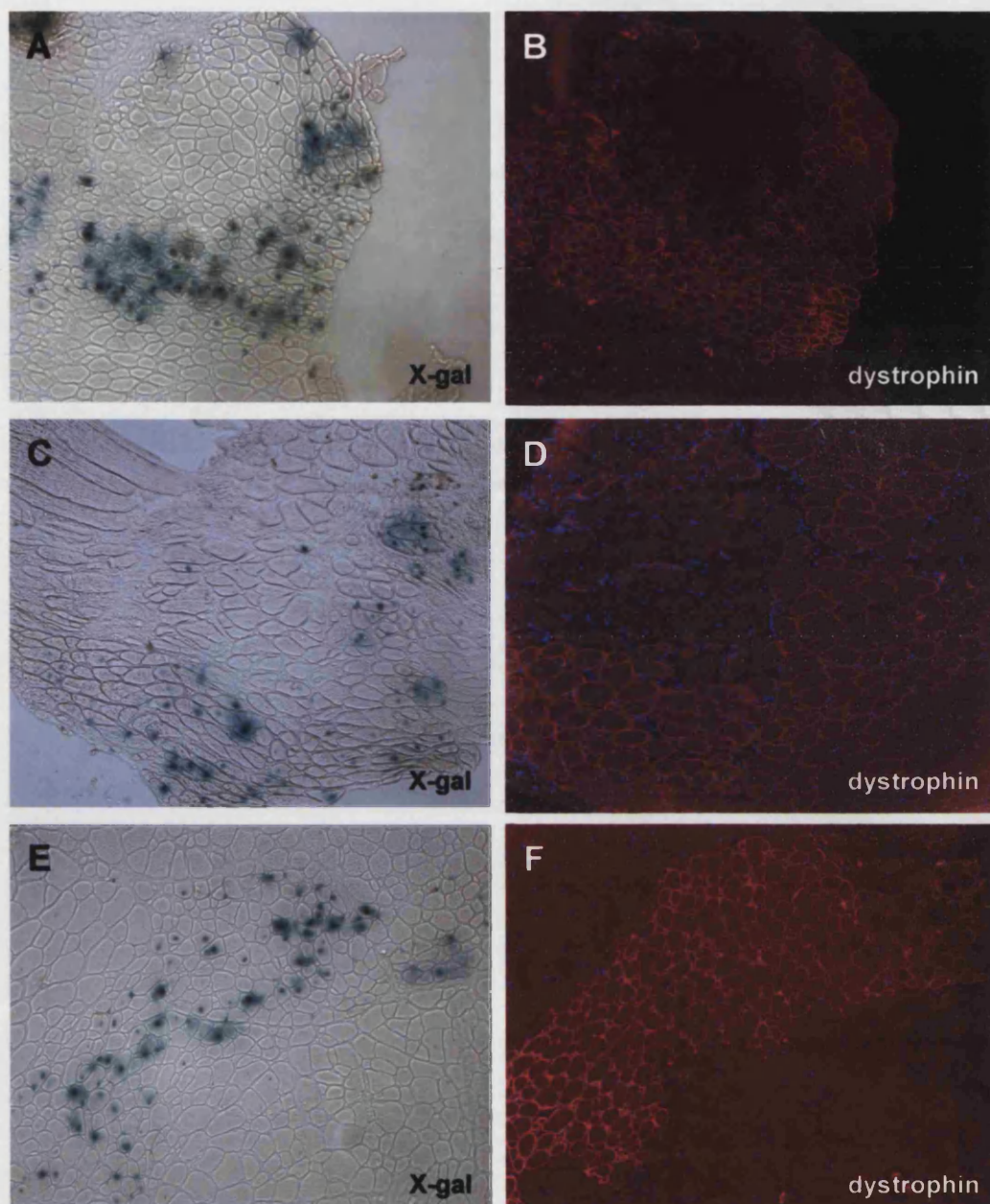


Fig. 3.6. Identification of donor-derived muscle formation in irradiated *mdx*-nude TA muscles engrafted with 5×10^4 primary myoblasts isolated from the EDL (A-B) soleus (C- D) or TA (E- F) muscles of adult 3F-*nLacZ*-2E mice. Donor-derived muscle is identifiable by X-gal staining to localise β -gal activity (A, C, E) and in serial sections, fluorescence immunohistochemistry for dystrophin protein, counterstained with DAPI (B, D, F). 3 week timepoint. Original magnification x100.

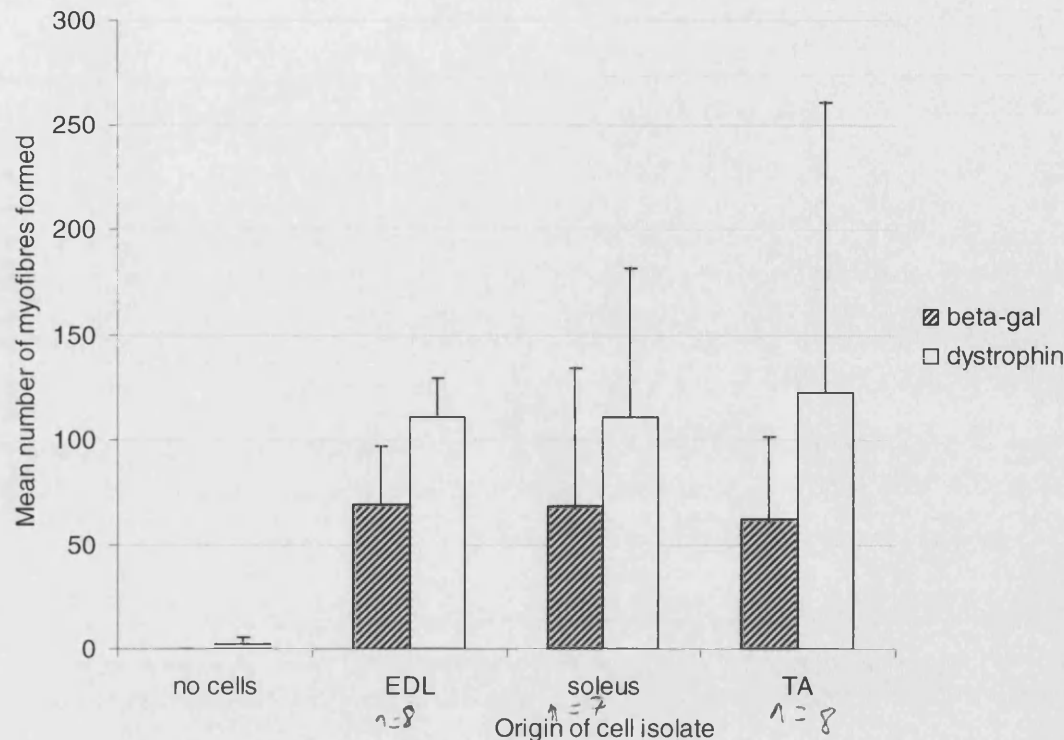


Fig. 3.7. Bar graph showing muscle formation by grafts of 5×10^4 primary desmin⁺ cells derived from different muscles of 42-day-old 3F-*nLacZ*-2E mice. After 3 weeks, engrafted muscles were removed and numbers of β -gal⁺ and dystrophin⁺ myofibres counted in representative mid-body sections. In each case, clusters of dystrophin⁺ myofibres contained both β -gal⁺ and β -gal⁻ myofibres. There was no statistically-significant difference (Mann-Whitney, $p > 0.05$) between the quantities of new muscle formed by cells derived from the different muscle groups. Error bars represent SD.

In each case, there was found to be a greater number of dystrophin⁺ myofibres than β -gal⁺ fibres (**Figs. 3.6 & 3.7**). The muscles of *mdx* mice contain small numbers of endogenous dystrophin⁺ 'revertant' myofibres that occur both singly and in small clusters (Hoffman *et al.* 1990, Lu *et al.* 2000). Increases in the number of revertant myofibres is partially suppressed after irradiation. After local irradiation at 21-days-old, 42-day-old *mdx* TA muscles typically contain less than twenty revertant myofibres, with a maximum of ten in a single cluster (personal communication, Q. Lu). The contribution of revertant myofibres cannot, therefore, wholly explain the deficit of β -gal expression within large clusters of dystrophin⁺ myofibres in engrafted muscles. The 3F-*nLacZ*-2E transgene becomes silenced in long-term cell cultures, both *in vitro* and *in vivo* (J. Morgan, personal communication). In all probability, the

greater proportion of dystrophin⁺ β -gal⁻ myofibres were therefore donor-derived, but failed to express the transgene.

There were no statistically significant differences (Mann-Whitney, $p \geq 0.05$) between the numbers of dystrophin⁺ myofibres or numbers of β -gal⁺ myofibres formed by grafts of cell isolates derived from the different muscles. In addition, the overall grafting efficiencies (the number of myofibres formed divided by number of myogenic cells grafted) were very similar (**Table 3.6**). These data show that MPC populations derived from adult EDL, soleus and TA muscles have equivalent myogenic potential after grafting into the same host environment.

3.4 Grafts of single myofibres derived from adult muscles

3.41 Aim

The objective of this study was to investigate the efficiency of muscle formation by grafts of single viable myofibres isolated from EDL, soleus and TA muscles.

3.42 Counts of Pax7⁺ satellite cells associated with single myofibres

This experiment aimed to determine the number of satellite cells associated with myofibres isolated from different muscles of adult mice, and thereby generate background data necessary for the analysis of subsequent grafting experiments. Single myofibres were isolated from the EDL, soleus and TA muscles (**Fig 2.1**) of 42 to 49-day-old 3F-*nLacZ*-2E mice (**2.21**). Myofibres from mouse 1 and mouse 2 were stained in X-gal solution to localise β -gal activity (**2.77**), and fluorescence immunocytochemistry was used to detect expression of Pax7, a marker of quiescent satellite cells (Seale *et al.* 2000, Zammit *et al.* 2004), and laminin, a major component of the basal lamina of myofibres (reviewed, Sanes 2003) (**Fig 3.8**). Satellite cells could therefore be identified by their absence of β -gal activity (Beauchamp *et al.* 2000), their expression of Pax7 protein (Seale *et al.* 2000, Zammit *et al.* 2004), and their anatomical position with respect to the basal lamina (Mauro 1961). Myofibres from mouse nos. 3, 4, 5, 6 and 7 were not stained in X-gal solution and were immunostained for expression of Pax7 protein only. Counts were made of the number of Pax7⁺ satellite cells associated with each myofibre (**Tables 3.2, 3.3, 3.4**).

The 3F transgene is expressed in the myonuclei of fast (glycolytic) myofibres (Kelly *et al.* 1995). Here, β -gal was found to be expressed ubiquitously in the myonuclei of myofibres isolated from the fast EDL and TA muscles of mouse 1 and mouse 2. A proportion of soleus myofibres stained only weakly, or not at all, in X-gal solution, demonstrating lack of fast myosin expression and implying a slow (oxidative) phenotype. The blue product of X-gal staining quenched fluorescence in myonuclei, hence in fast myofibres only the nuclei of satellite cells were strongly labelled by DAPI (**Fig. 3.8**). Since confocal microscopy was not used for this study, the position of some Pax7⁺ satellite cells was equivocal with respect to the basal lamina, as visualised by laminin immunostaining. However, the basal lamina appeared to

overlay all Pax7⁺ cells whose position in the plane of view made unequivocal anatomical localisation possible (**Fig. 3.8**).

Previous studies have identified the number of satellite cells associated with single myofibres isolated from *Myf5^{nLacZ/+}* EDL muscles to be 4.4 to 4.5 as identified by immunostaining for M-cadherin, CD34 or Myf5- β -gal fusion protein (Beauchamp *et al.* 2000) or 7.62 as identified by expression of Myf5- β -gal fusion protein using X-gal staining (Zammit *et al.* 2002). In 3F-*nLacZ*-2E EDL myofibres stained in X-gal, the number of nuclei not expressing β -gal but identifiable by DAPI immunofluorescence has been calculated as 5.56 (Beauchamp *et al.* 2000). Thus counts made in this study of 7.23 (± 2.19) Pax7⁺ satellite cells per EDL myofibre (**Table 3.2**) broadly agree with data previously generated by other workers using different markers.

	No. of myofibres	Mean Pax7 ⁺ satellite cells
mouse 1	25	6.64 (± 3.66)
mouse 2	18	6.39 (± 3.31)
mouse 3	15	11.07 (± 3.69)
mouse 4	8	5.63 (± 2.77)
mouse 7	12	6.33 (± 2.10)
Total	77	7.23 (SE ± 2.19)

Table 3.2. The numbers of Pax7⁺ satellite cells associated with single myofibres isolated from the EDL muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice.

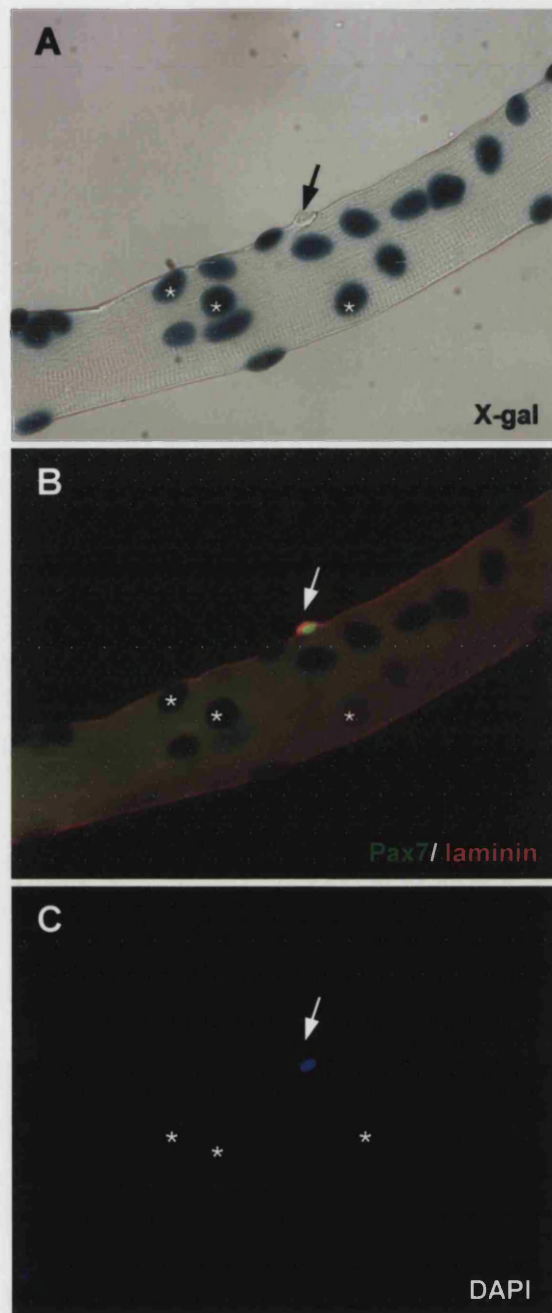


Fig. 3.8. Identification of satellite cells associated with single myofibres isolated from 3F-*nLacZ*-2E muscles. Arrows mark the nucleus of a satellite cell and asterisks mark examples of myonuclei. Staining in X-gal solution localises β -gal activity to myonuclei (A). The nucleus of a satellite cell does not express β -gal (A) but is detectable by Pax7 immunocytochemistry (B) and DAPI staining (C), and is beneath the basal lamina as visualised by laminin immunocytochemistry (B). Original magnification x400.

In single myofibres isolated from the soleus muscles of *Myf5^{nLacZ/+}* mice, a previous study identified the number of satellite cells identified by expression of Myf5- β -gal fusion protein to be 26.23 per myofibre (Zammit *et al.* 2002). In the present study, Pax7 immunostaining of 3F-*nLacZ*-2E soleus single myofibres yielded a slightly lower count of 22.08 (± 5.11) satellite cells per myofibre (**Table 3.3**). This difference could be attributable to heterogeneity of antigen expression, inter-strain variability (though the gene-targeted *Myf5^{nLacZ}* and the 3F-*nLacZ*-2E transgenic were generated on the same wild-type background, C57 Bl/6), or the relatively small sample number in the present study.

	No. of myofibres	Mean Pax7 ⁺ satellite cells
mouse 1	18	24.06 (± 10.71)
mouse 2	13	24.08 (± 8.55)
mouse 5	9	15.22 (± 5.24)
Total	40	22.08 (SE ± 5.11)

Table 3.3. The numbers of Pax7⁺ satellite cells associated with single myofibres isolated from the soleus muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice.

There are no published data describing the number of satellite cells associated with TA single myofibres. Unpublished counts on *Myf5^{nLacZ/+}* TA myofibres found the mean number of satellite cells expressing Myf5- β -gal fusion protein to be 13.7 per myofibre (personal communication, P.S. Zammit). In this experiment, the number of satellite cells as identified by Pax7 expression on 3F-*nLacZ*-2E TA myofibres was rather lower, at 9.66 (± 0.02) (**Table 3.4**). Whilst the sample number was not big, the low standard error between the three animals implies that the data are representative.

	No. of myofibres	Mean Pax7 ⁺ satellite cells
mouse 1	16	9.75 (± 5.74)
mouse 2	24	9.50 (± 4.88)
mouse 6	10	9.90 (± 4.95)
Total	50	9.66 (SE ± 0.02)

Table 3.4. The numbers of Pax7⁺ satellite cells associated with single myofibres isolated from the TA muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice.

The numbers of Pax7⁺ satellite cells associated with single myofibres derived from different muscles are shown in **Fig. 3.9**. The Mann-Whitney test was used to compare counts of Pax7⁺ satellite cells associated with single myofibres derived from the three different muscles. The differences between numbers of EDL and soleus satellite cells, and between numbers of soleus and TA satellite cells, were found to be extremely significant ($p < 0.0001$), and the difference between numbers of EDL and TA satellite cells was found to be significant ($p = 0.0139$).

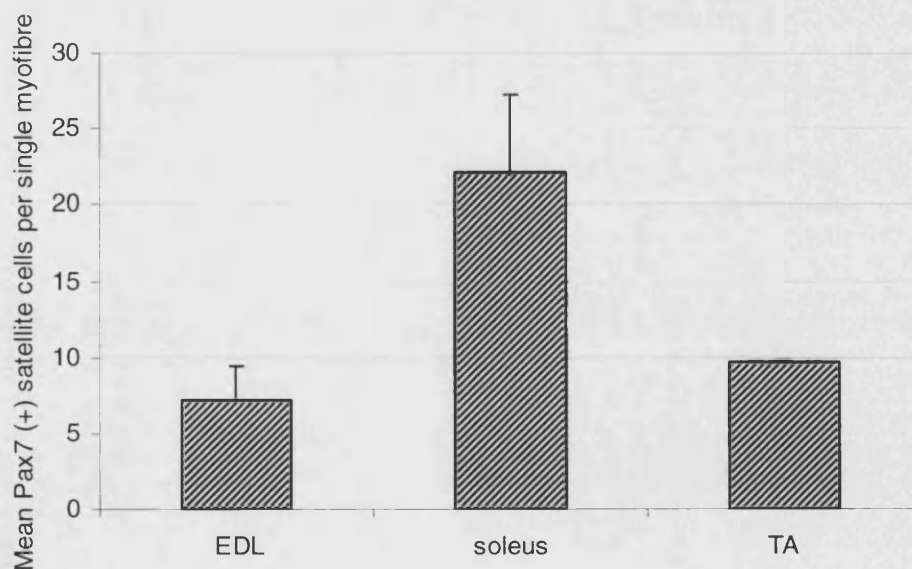


Fig. 3.9. Counts of Pax7⁺ satellite cells associated with myofibres isolated from the EDL, soleus and TA muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice. Error bars represent SE.

3.43 Preparation and grafting of single myofibres

Single myofibres were isolated from the EDL, soleus and TA muscles (**Fig. 2.1**) of 42 to 49-day-old young adult 3F-*nLacZ*-2E mice as described (**2.21**). Individually-selected perfect myofibres were washed by passing through 3 dishes of warmed serum-free medium and then kept in small groups of about 20-30 to prevent entanglement. A maximum of 30 minutes was allowed between myofibre preparation and transfer to the procedure room for grafting (**2.55**). Usually, the use of small groups of 4-8 mice meant that the grafting procedure could be completed in less than 1 h. A small proportion of myofibres did not survive outside the incubator for this period of time and developed kinks or areas of hypercontraction, and were immediately discarded. To prepare the graft, a single myofibre was drawn into a glass needle such that it laid undamaged and unbent within the body of the needle, confirmed by visual inspection. The graft was then inserted into the recipient muscle under microscopic observation. Any fluid expelled from the injection site was re-drawn into the needle and re-inserted. To confirm that the myofibre had exited the needle and not become stuck to the inside, or hypercontracted and lodged in its tip, the needle was flushed out several times under the microscope. If this process yielded a damaged myofibre, a fresh intact myofibre was drawn up and the grafting process repeated. This process aimed, as far as practicably possible, to graft presumptively quiescent satellite cells that had had minimal exposure to the tissue culture environment, and were maintained within their endogenous niche below the basal lamina of intact myofibres. Immediately after isolation, about 10% of satellite cells in single myofibre preparations are activated as defined by immunohistochemical detection of MyoD protein. After a 6 h exposure to serum mitogens, 80-90% become activated by the same criterion (Zammit *et al.* 2002). It is therefore likely that by the time the graft was inserted into the host site, it contained both quiescent satellite cells and cells that were in the early stages of activation.

Single myofibre grafts were inserted into the TA muscles (**2.55**) of 24-day-old *mdx*-nude mice, which had been exposed to 18 Gy irradiation 3 days previously (**2.53**). Engrafted muscles were removed after 3 weeks and processed for immunohistochemistry (**2.61**). 3 serial 7 μ m sections were collected at 100 μ m intervals throughout the entire TA muscle body (about 30-40 levels) (**2.62**). The first

set of sections was stained in X-gal solution to localise β -gal activity (2.77). The second set of serial sections was immunohistochemically stained for dystrophin protein (2.76). The maximum number of β -gal⁺ and dystrophin⁺ myofibres were counted in single serial sections. The number of dystrophin⁺ myofibres was usually greater than, and never less than, the number of β -gal⁺ myofibres. As previously discussed (3.42), this difference is probably partly attributable to the presence of endogenous revertant myofibres and partly to the *nLacZ* transgene becoming silenced after extensive proliferation. Typically, the products of grafts formed clusters of dystrophin⁺ myofibres which included both β -gal⁺ and β -gal⁻ myofibres. The numbers of dystrophin⁺ myofibres probably slightly over-estimate, whilst the numbers of β -gal⁺ myofibres almost certainly under-estimate, the quantity of donor-derived muscle formation. Hence both sets of data are presented together.

3.44 The myogenic potential of EDL single myofibre grafts

A total of 34 single myofibre grafts derived from the EDL muscles of four donor animals were carried out. 11/34 (32%) of engrafted muscles contained both dystrophin expression and β -gal activity (Figs. 3.10 & 3.11). The quantity of donor-derived muscle ranged from 2 dystrophin⁺ myofibres of which 1 was β -gal⁺, to 189 dystrophin⁺ myofibres of which 72 were β -gal⁺. Dystrophin immunohistochemistry was not carried out on muscles that had no detectable β -gal activity. In total, an estimated 238 EDL satellite cells (based on 34 EDL single myofibres with an average of 7 associated satellite cells) were grafted in this study, generating a total of 298 β -gal⁺ myofibres and 526 dystrophin⁺ myofibres. Therefore the absolute efficiency of muscle formation was 1.25 β -gal⁺ myofibres and 2.21 dystrophin⁺ myofibres per grafted satellite cell. If grafts that generated no donor-derived muscle are excluded from the calculation, the efficiency was 3.87 β -gal⁺ myofibres and 6.26 dystrophin⁺ myofibres per satellite cell. Similarly, in the single graft that made the most muscle, the estimated efficiency was 10.14 β -gal⁺ myofibres and 27.00 dystrophin⁺ myofibres per satellite cell (Table 3.6).

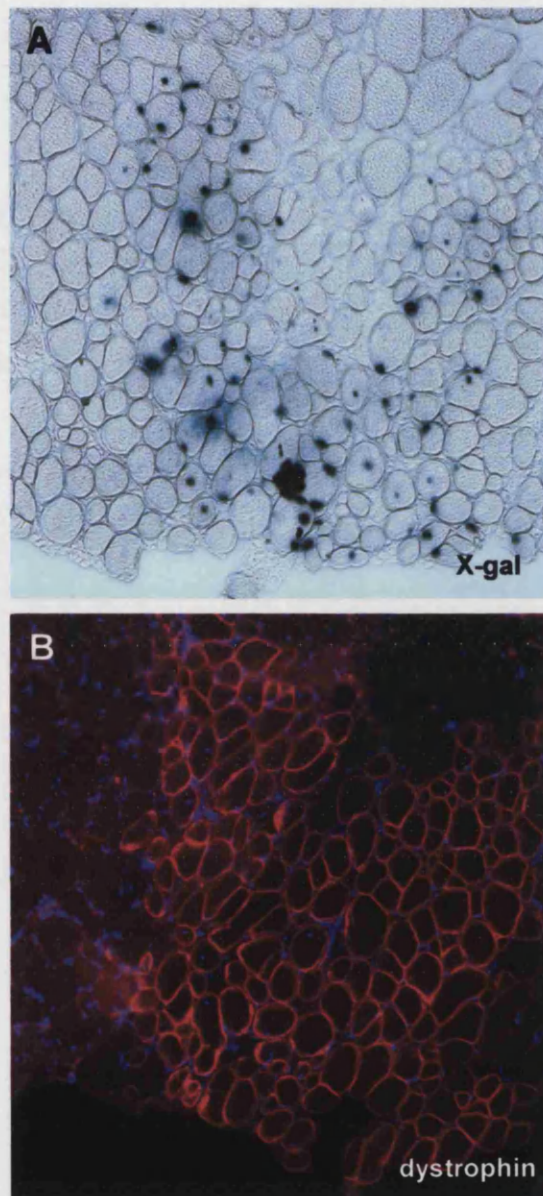


Fig. 3.10. Identification of donor-derived muscle formation in an irradiated *mdx*-nude TA muscle engrafted with a single viable myofibre isolated from the EDL muscle of a 42-day-old adult 3F-*nLacZ*-2E mouse. Donor-derived muscle is identifiable by X-gal staining to localise β -gal activity (A), and in a serial section, fluorescence immunohistochemistry for dystrophin protein, counterstained with DAPI (B). 3 week timepoint. Original magnification x100.

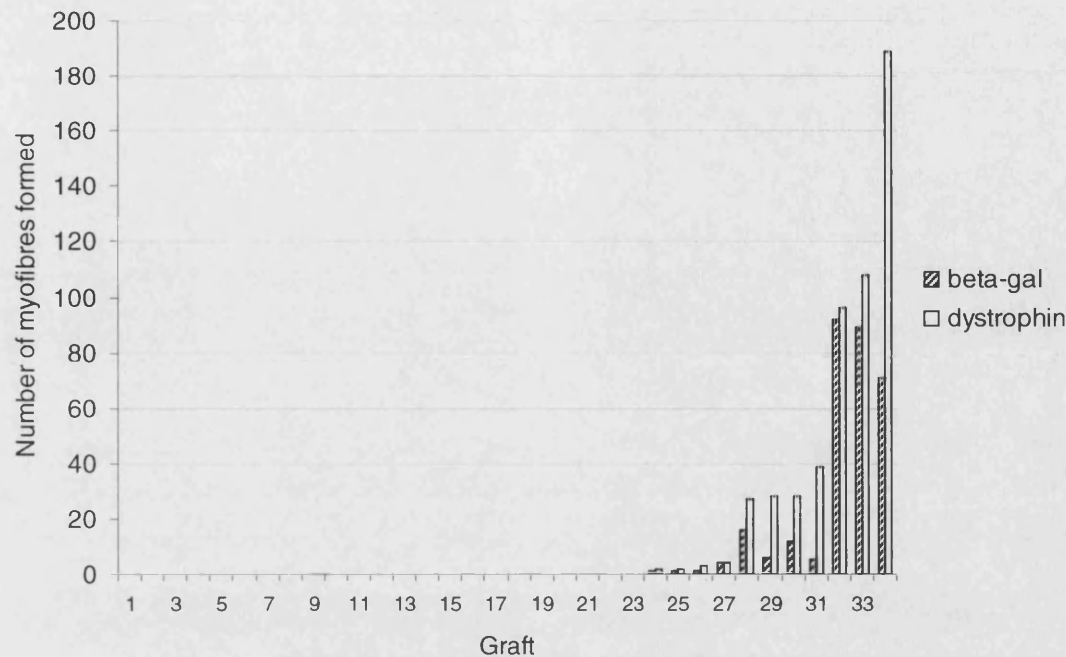


Fig. 3.11. Histogram showing muscle formation by individual grafts of single myofibres derived from the EDL muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice. Ranked in order of the number of dystrophin⁺ myofibres. Muscles were harvested 3 weeks post-grafting. Counts were made of the maximum numbers of dystrophin⁺ and β -gal⁺ myofibres in single serial sections of engrafted muscles. 11/34 (32%) of engrafted muscles contained both β -gal activity and dystrophin expression. Clusters of dystrophin⁺ myofibres contained both β -gal⁺ and β -gal⁻ myofibres. Engrafted muscles with no detectable β -gal activity were not stained for dystrophin. Pooled data from 4 experiments.

Each TA myofibre has approximately 600 myonuclei (J.D. Rosenblatt & T.A Partridge, unpublished data). Although many myofibres expressing donor markers are likely to have been host/donor mosaics (Partridge *et al.* 1978, Yang *et al.* 1997, Blaveri *et al.* 1999), a conservative estimate of a 25% donor contribution would suggest that within the 3 week time-course, some grafted EDL satellite cells had the potential to clonally generate several thousand differentiation-competent progeny.

The previous study carried out by Heslop under similar conditions found that grafts of EDL myofibres generated muscle in only 13% of cases, but that almost all successful grafts generated >100 new myofibres (L. Heslop, PhD Thesis 2001). In contrast to this finding, the present study found that 32% of EDL myofibre grafts generated

identifiable donor-derived products. The quantity of new muscle formed was highly variable, with only a small number of grafts generating >100 myofibres (**Fig. 3.11**).

3.45 The myogenic potential of soleus single myofibre grafts

A total of 20 grafts derived from three donor animals were carried out. 15/20 (75%) of grafts generated donor-derived muscle which ranged in quantity from 2 dystrophin⁺ myofibres of which 1 was β -gal⁺, to 159 dystrophin⁺ myofibres of which 142 were β -gal⁺. Since slow myofibres do not express β -gal at detectable levels, it was not known whether the products of soleus slow myofibre grafts would be identifiable by β -gal activity. For this reason, fluorescence immunohistochemistry for dystrophin protein was carried on all engrafted muscles. However, large numbers of dystrophin⁺ myofibres were only identified in association with β -gal activity (**Figs. 3.12 & 3.13**), showing that all successful grafts generated at least some new myofibres that expressed fast myosin.

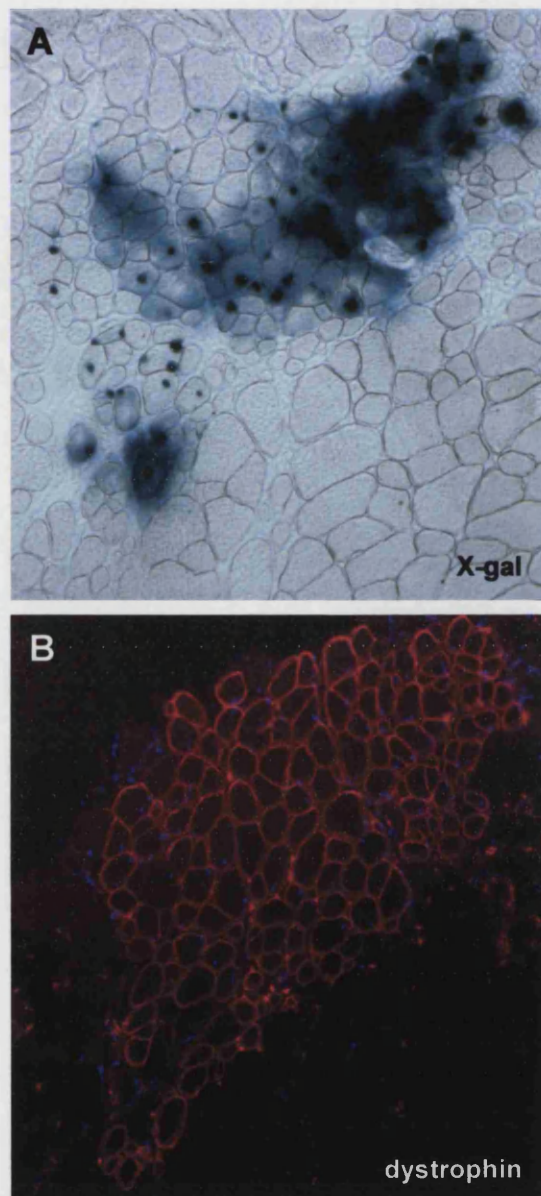


Fig. 3.12. Identification of donor-derived muscle formation in an irradiated *mdx*-nude TA muscle engrafted with a single viable myofibre isolated from the soleus muscle of a 42-day-old adult 3F-*nLacZ*-2E mouse. Donor-derived muscle is identifiable by X-gal staining to localise β -gal activity (A), and in a serial section, fluorescence immunohistochemistry for dystrophin protein, counterstained with DAPI (B). 3 week timepoint. Original magnification x100.

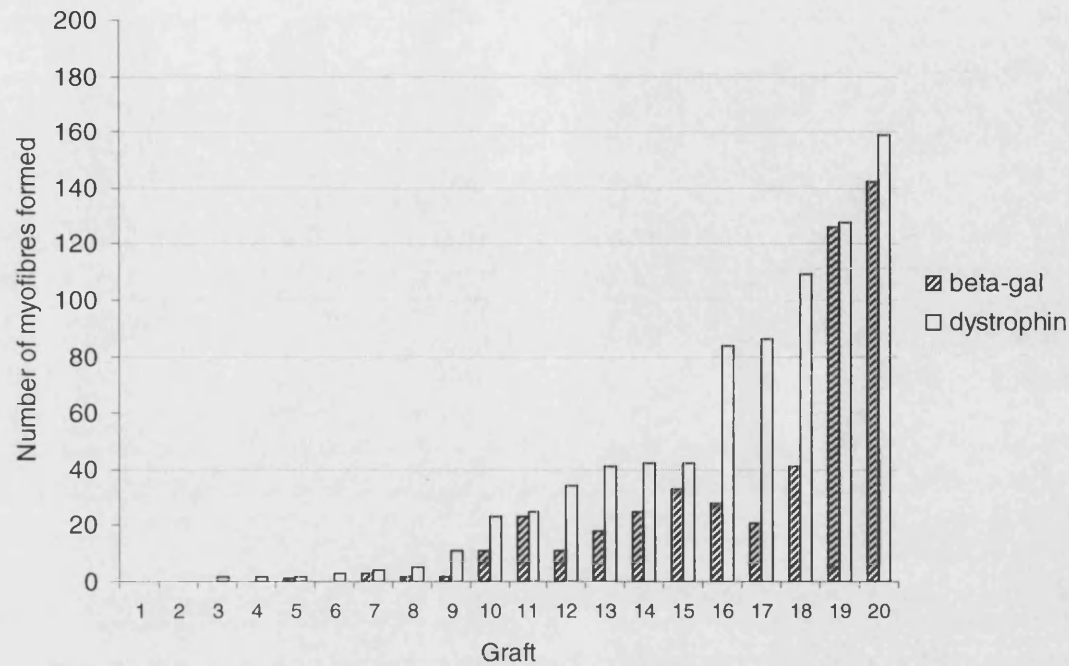


Fig. 3.13. Histogram showing muscle formation by individual grafts of single myofibres derived from the soleus muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice. Ranked in order of the number of dystrophin⁺ myofibres. Muscles were harvested 3 weeks post-grafting. Counts were made of the maximum numbers of dystrophin⁺ and β -gal⁺ myofibres in single serial sections of engrafted muscles. 15/20 (75%) of engrafted muscles contained both β -gal activity and dystrophin expression. Clusters of dystrophin⁺ myofibres contained both β -gal⁺ and β -gal⁻ myofibres. Pooled data from 3 experiments.

The Heslop study found that 34% of soleus myofibre grafts generated new muscle. The number of new myofibres formed ranged from 8 to 200 and had an approximately normal distribution. Whilst the results of the present study differ from the finding of Heslop in that grafted myofibres generated new muscle an increased efficiency of 75%, they concur with Heslop in similarly showing a large range in the numbers of new myofibres formed (**Fig. 3.13**).

3.46 The myogenic potential of TA single myofibre grafts

A total of 42 grafts from three donor animals were carried out. 14/42 (33%) of engrafted muscles contained donor-derived myofibres identifiable by β -gal activity. The quantities of donor-derived muscle formed ranged from 1 to 11 β -gal⁺ myofibres, and had an approximately normal distribution (**Fig 3.15**). Due to prepared slides being lost, only 6/42 engrafted muscles were stained for dystrophin protein. Of these, 2/6 engrafted muscles respectively contained 11 dystrophin⁺ myofibres of which 2 were β -gal⁺, and 7 dystrophin⁺ myofibres of which 4 were β -gal⁺ (**Fig. 3.14**). The results of previously-described experiments suggest that alone, counts of β -gal⁺ myofibres are an under-estimate of the total donor contribution to engrafted muscles. Notwithstanding, it is notable that out of a total of 42 grafts, no TA myofibre graft generated more than 11 new myofibres. These data suggest that satellite cells with the potential to generate large amounts of differentiated tissue are absent from the TA muscle. Alternatively, they may occur at a frequency too low to be detectable by this protocol, with which only small total numbers of cells can be analysed.

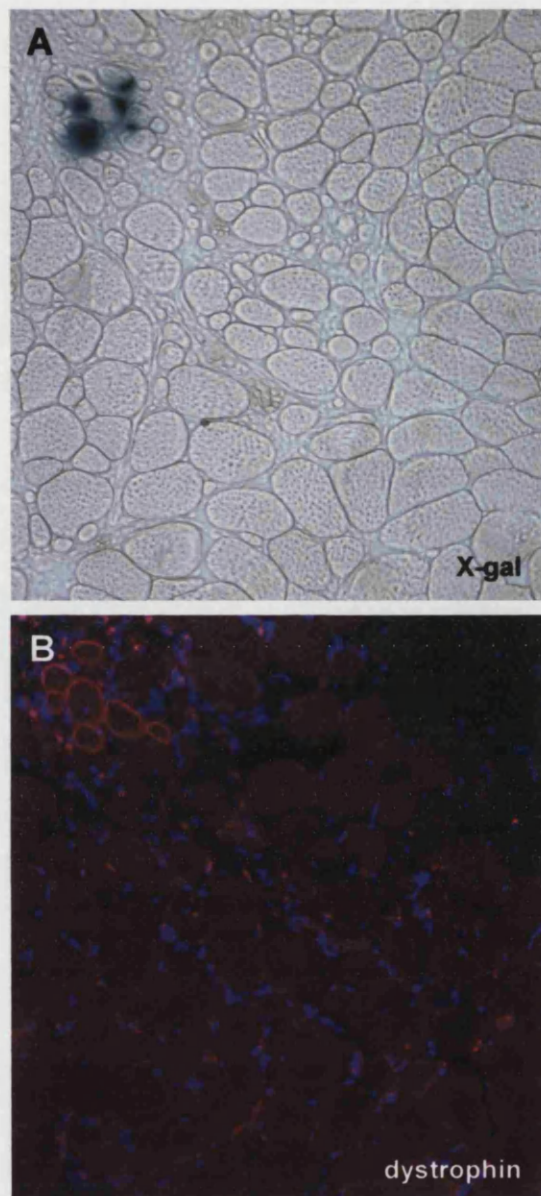


Fig. 3.14. Identification of donor-derived muscle formation in an irradiated *mdx*-nude TA muscle engrafted with a single viable myofibre isolated from the soleus muscle of a 42-day-old adult 3F-*nLacZ*-2E mouse. Donor-derived muscle is identifiable by X-gal staining to localise β -gal activity (A), and in a serial section, fluorescence immunohistochemistry for dystrophin protein, counterstained with DAPI (B). 3 week timepoint. Original magnification x100.

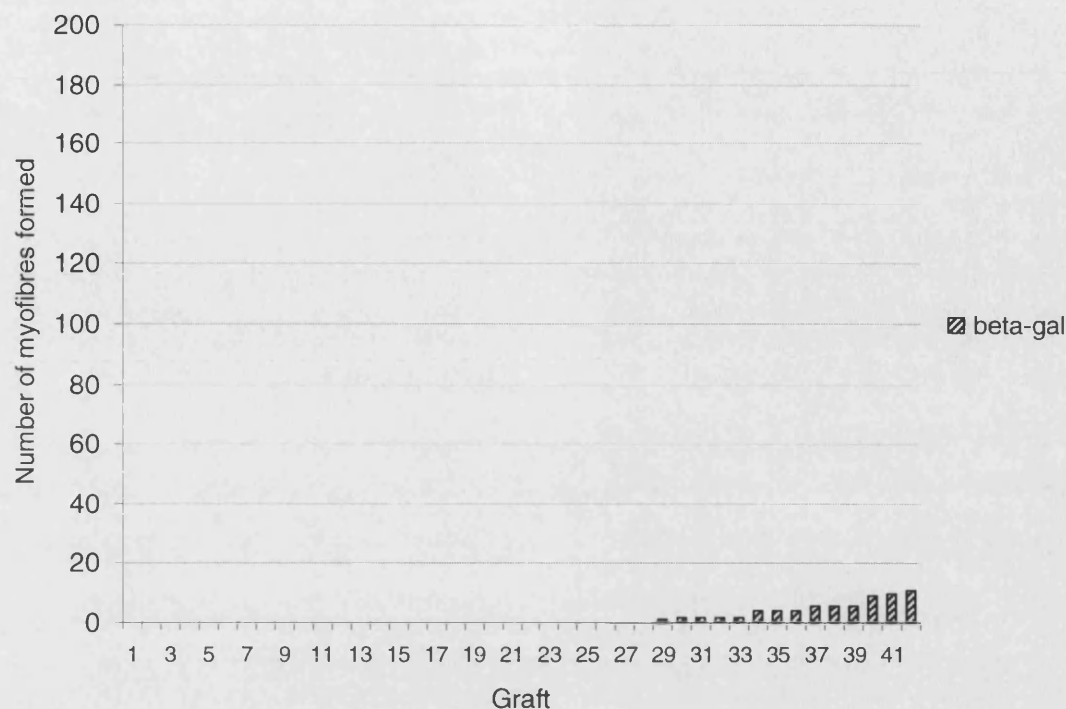


Fig. 3.15. Histogram showing muscle formation by individual grafts of single myofibres derived from the TA muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice. Ranked in order of the number of β -gal⁺ myofibres. Muscles were harvested 3 weeks post-grafting. Counts were made of the maximum number β -gal⁺ myofibres in a single sections of each engrafted muscle. 14/42 (33%) of engrafted muscles contained β -gal activity. Pooled data from 3 experiments.

3.47 Summary: comparison of the myogenic potential of the satellite cell populations of different muscles

Since myofibres isolated from different muscles have different numbers of associated satellite cells, it might be predicted that they would generate different quantities of muscle after grafting. However, despite the fact that soleus myofibres have about three times as many satellite cells as EDL myofibres (3.42), the Heslop study found that successful soleus myofibre grafts only generated about a third as many new myofibres as successful EDL myofibre grafts (L. Heslop, PhD thesis 2001). In the present study, however, successful grafts of EDL and soleus myofibres generated similar mean amounts of β -gal⁺ myofibres and dystrophin⁺ myofibres, but successful grafts of TA myofibres generated substantially smaller numbers of β -gal⁺ myofibres

(counts of dystrophin⁺ myofibres were not taken for most TA myofibre engrafted muscles) (**Table 3.5**). Thus these data show that the amount of new muscle formed is not directly proportional to the number of cells present in the graft. However, the frequency of successful grafts (the number of grafts generating at least 1 β -gal⁺ myofibre divided by the total number of grafts), did appear to be proportional to the number of satellite cells originally present in the graft, since soleus myofibres, which have 22 associated satellite cells (**3.42**), generated muscle more than twice as frequently as EDL or TA myofibres, which have 7 and 10 associated satellite cells respectively (**3.42**). It is possible that technical failure was responsible for the failure of a proportion of grafts, but there is no obvious reason why the failure rate should have been lower for soleus myofibre grafts than it was for EDL or TA myofibre grafts. Assuming that some EDL and TA myofibres were successfully grafted, but generated no muscle, these data suggest that only a proportion of satellite cells were recruited into an active myogenic program.

	EDL single myofibre	soleus single myofibre	TA single myofibre
Satellite cells per graft ^a	7	22	10
No. of grafts	34	20	42
Percentage of successful ^b grafts	32%	75%	33%
Mean β -gal ⁺ myofibres formed by successful grafts	27.1 (\pm 37.2)	32.5 (\pm 43.1)	4.9 (\pm 3.2)
Mean dystrophin ⁺ myofibres formed by successful grafts	47.8 (\pm 59.4)	53.5 (\pm 49.0)	ND

^a Mean Pax7⁺ satellite cells per single myofibre (**3.41**). ^b Grafts with ≥ 1 β -gal⁺ nuclei.

Table 3.5. Comparison of the quantities of muscle formed by grafts of single myofibres derived from the EDL, soleus and TA muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice. ND= not done.

When the data are expressed as the total number of myofibres formed per total grafted satellite cells, EDL satellite cells were found to generate new muscle with slightly higher overall efficiency than soleus satellite cells. Both EDL and soleus satellite cells generated new muscle with much greater efficiency than TA satellite cells (**Table 3.6**). In addition, the maximum grafting efficiency (the maximum number of new myofibres formed by a single graft divided by the number of satellite cells present in the graft) was much greater for EDL single myofibre grafts than for either soleus or TA myofibre grafts (**Table 3.6**).

The numbers of β -gal⁺ myofibres formed by grafts of single myofibres derived from EDL, soleus and TA muscles were compared using the Kruskal-Wallis test (see appendix). The differences between the three groups were found to be extremely significant ($p=0.0003$). The differences were further analysed using Dunn's multiple comparisons test (see appendix). This showed that the difference in muscle formation between grafts of EDL myofibres and grafts of soleus myofibres was significant ($p<0.01$) and the difference in muscle formation between grafts of soleus myofibres and grafts of TA myofibres was very significant ($p<0.001$). There was no statistically-significant difference ($p>0.05$) between muscle formation by grafts of EDL myofibres and muscle formation by grafts of TA myofibres. However, differences between these two data sets are clearly apparent both from plots (**Figs. 3.11 & 3.15**), and from the mean amounts of muscle formed (**Table 3.5**). The absence of statistical significance is probably due to the large numbers of zeros in both data sets.

The results of this study show that satellite cells in single myofibre grafts have a stem cell-like myogenic potential and exhibit patterns of behaviour which vary according to their muscle of origin.

	SF1K clone	SF1K clone	SF1K clone	SF1K clone	EDL primary	soleus primary	TA primary	EDL myofibre	soleus myofibre	TA myofibre
No. myogenic cells per graft	5×10^2	5×10^3	5×10^4	5×10^5	5×10^4	5×10^4	5×10^4	7 ^a	22 ^a	10 ^a
No. of grafts	5	4	4	6	8	7	8	34	20	42
Total myogenic cells in all grafts	2.5×10^3	2×10^4	2×10^5	3×10^6	4×10^5	3.5×10^5	4×10^5	238	440	420
Total β -gal ⁺ myofibres formed by all grafts	13	15	351	1400	552	480	499	298	487	69
Total dystrophin ⁺ myofibres formed by all grafts	ND	ND	ND	ND	886	775	980	526	802	ND
Overall efficiency of all grafts ^b	0.0052	0.0008	0.0018	0.0005	0.0022	0.0021	0.0029	2.2108	1.8227	0.1643
Max. β -gal ⁺ myofibres formed by single graft	12	8	265	453	167	228	122	71	142	11
Max. dystrophin ⁺ myofibres formed by a single graft	ND	ND	ND	ND	420	338	523	189	159	ND
Max. efficiency of a single graft ^b	0.0240	0.0016	0.0053	0.0009	0.0084	0.0068	0.0105	27.0000	7.2273	1.1000

^a Mean Pax7⁺ satellite cells per single myofibre (**3.41**). ^b Efficiency= the total donor-derived myofibres formed divided by the total myogenic cells grafted.

Table. 3.6. Comparison of the efficiency of muscle formation generated from adult myogenic cells grafted into irradiated *mdx*-nude TA muscles. Engrafted muscles were removed after 3 weeks. ND= Not done.

3.48 Fibre types formed by single myofibre grafts

All successful grafts of single myofibres derived from 3F-*nLacZ*-2E soleus muscles generated myofibres that expressed the *nLacZ* transgene, implying that the satellite cells associated with slow myofibres were able to express fast myosin after differentiation (3.44). To further investigate whether the satellite cells in single myofibre grafts remained committed to the fibre-type of their parent myofibre, serial sections from some engrafted muscles were stained with an antibody that recognised fast myosin isoforms and an antibody that recognised slow myosin (2.76, Table 2.1). Staining of sections from control wild-type muscles showed that the anti-fast myosin antibody bound to all myofibres in the glycolytic TA and EDL muscles, and to about 60% of myofibres in the oxidative soleus muscle (Fig. 3.16). The anti-slow myosin antibody bound to about 50% of myofibres in the oxidative soleus muscle and to occasional myofibres in glycolytic EDL and TA muscles (Fig. 3.16). In the TA muscle, most slow-myosin⁺ myofibres were present in the superficial part. Therefore, as expected, control TA and EDL muscles contained mainly fast myofibres with a few slow/fast hybrids, and soleus muscles contained fast myofibres, slow/fast hybrids and slow myofibres.

In total, 11 muscles successfully-engrafted with soleus myofibres, 2 muscles engrafted with EDL myofibres, and 2 muscles engrafted with TA myofibres were examined. Whilst 7/15 grafted myofibres generated fast myosin isoforms only, 8/15 grafted myofibres generated both fast and slow myosin isoforms (Table 3.7, Fig. 3.16). All individual myofibres generated by all grafts expressed fast myosin, suggesting that in many fibres, expression of the *nLacZ* transgene did not faithfully report expression of the 3F gene. Expression of slow myosin was always localised to slow/fast hybrid myofibres; no exclusively slow myofibres were observed in any of the 15 engrafted muscles examined. With one exception, fast/slow hybrid myofibres formed <6% of the total myofibres derived from each graft (Table 3.7). These data suggest that whilst discrete satellite cell populations are capable of contributing to myofibres with different patterns of myosin isoform expression, they do not remain committed to the expression profile of their fibre type of origin, but probably adopt the expression profile of the engrafted muscle. The data do not exclude the possibility that the satellite cells which contributed to slow/fast hybrid myofibres were

themselves derived from parent myofibres which expressed slow myosin. It was therefore not possible to resolve whether the products of grafted satellite cells acquired the fibre-type profile of the host tissue and were not committed to expressing particular myosin isoforms (Hughes & Blau 1992, Bonavaud *et al.* 2001), or whether they had a degree of prior commitment to a particular pattern of myosin isoform expression (Rosenblatt *et al.* 1996), as has been previously suggested by other studies.

Single myofibre graft	Total dystrophin ⁺ myofibres	dystrophin ⁺ fast myosin ⁺ slow myosin ⁻	dystrophin ⁺ fast myosin ⁻ slow myosin ⁺	dystrophin ⁺ fast myosin ⁺ slow myosin ⁺
soleus 1	86	97.7%	0	2.3%
soleus 2	34	100%	0	0
soleus 3	84	100%	0	0
soleus 4	109	100%	0	0
soleus 5	159	99.4%	0	0.6%
soleus 6	41	100%	0	0
soleus 7	42	100%	0	0
soleus 8	23	95.7%	0	4.4%
soleus 9	25	96.0%	0	4.0%
soleus 10	128	96.9%	0	3.1%
soleus 11	42	95.2%	0	4.8%
EDL 1	96	82.3%	0	17.7%
EDL 2	189	94.7%	0	5.3%
TA 1	11	100%	0	0
TA 2	7	100%	0	0

Table 3.7. Percentages of fast, slow and fast/slow hybrid myofibres in the products of soleus, EDL and TA single myofibre grafts into TA muscles.

Unexpectedly, the two myofibre grafts which generated the greatest proportions of slow myosin⁺ myofibres were those derived from EDL muscles (**Table 3.7**), suggesting that EDL satellite cells might have a greater degree of plasticity with regards to myosin isoform expression.

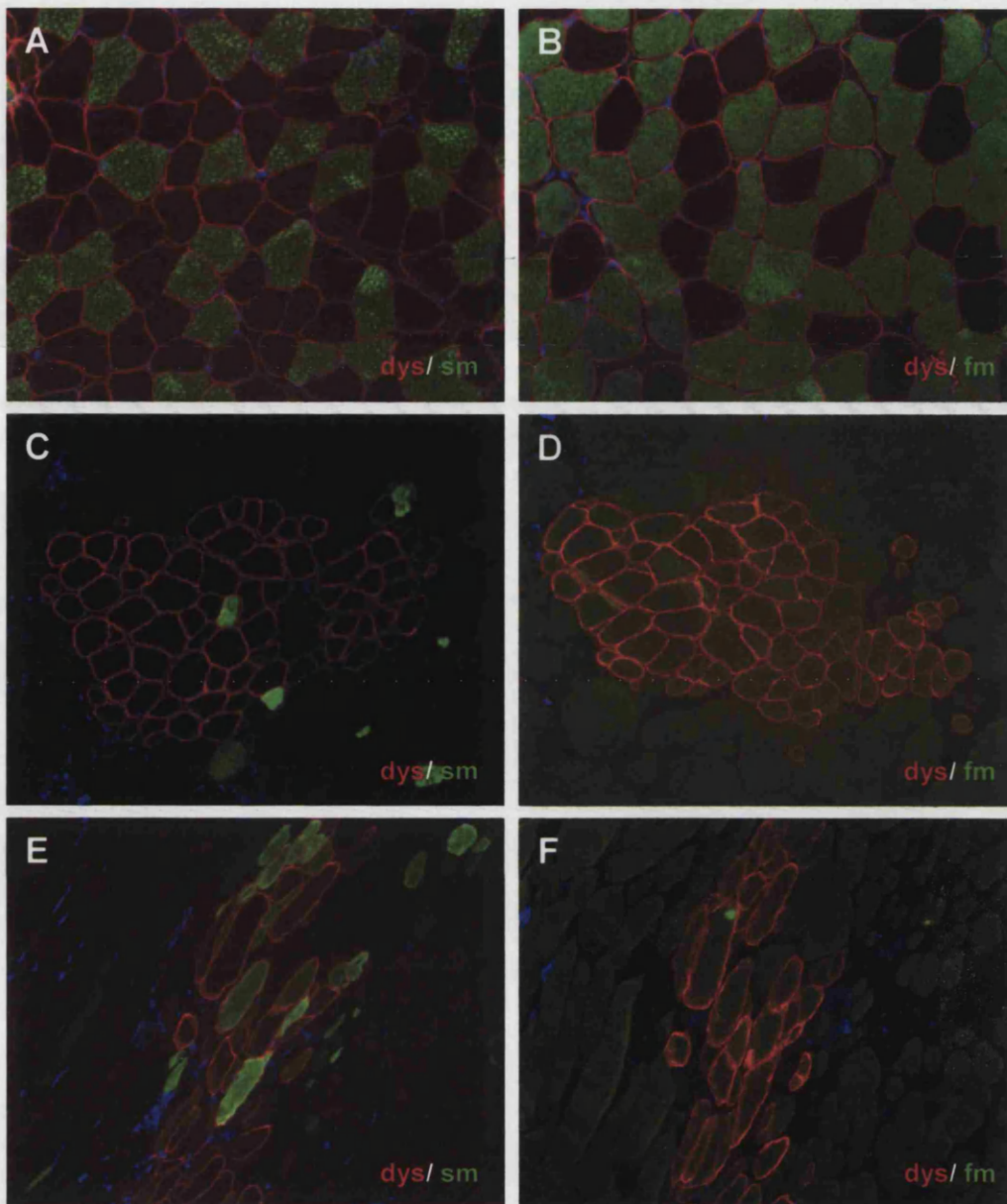


Fig. 3.16. Expression of fast and slow myosin isoforms in muscle generated from grafts of single myofibres. Immunohistochemistry for dystrophin and slow myosin (A, C, E) and dystrophin and fast myosin (B, D, F). A and B are control sections of wild-type soleus muscle (different areas of same muscle). C-F are serial sections of *mdx*-nude TA muscles engrafted with a single myofibre derived from a 3F-*nLacZ*-2E soleus (C-D) or EDL (E-F) muscle. All myofibres derived from single myofibre grafts expressed fast myosin (D, F); some also co-expressed slow myosin (C, E). Original magnification x200.

3.49 The myogenic potential of EDL single myofibres in long-term grafts

When examined at the 3 week timepoint, muscles engrafted with EDL single myofibres were found to contain numbers of donor-derived myofibres which had a very large range (**Fig. 3.11**). Hypothetically, the large range could be attributable to asynchrony of inter-graft satellite cell activation, whereby the smaller muscle colonies represent earlier stages of clonal expansion. The objective of this experiment was to test this hypothesis by analysing myofibre-engrafted muscles at a later timepoint.

Single myofibre grafts were inserted into 16 pre-irradiated TA muscles of *mdx*-nude host mice (**2.53, 2.55**). Engrafted TA muscles were removed after 12 weeks, and three serial 7 μm sections taken at 100 μm intervals throughout each entire muscle body (**2.61, 2.62**). In the first set of sections, X-gal staining was used to localise β -gal activity (**2.77**), and in the second set, dystrophin protein was detected immunohistochemically (**2.76**). For each engrafted muscle, counts were made of the maximum number of β -gal⁺ and dystrophin⁺ myofibres present in one section from the same level (**Fig. 3.17**).

9/16 (56%) of engrafted muscles contained both β -gal activity and dystrophin expression (**Fig. 3.18**).

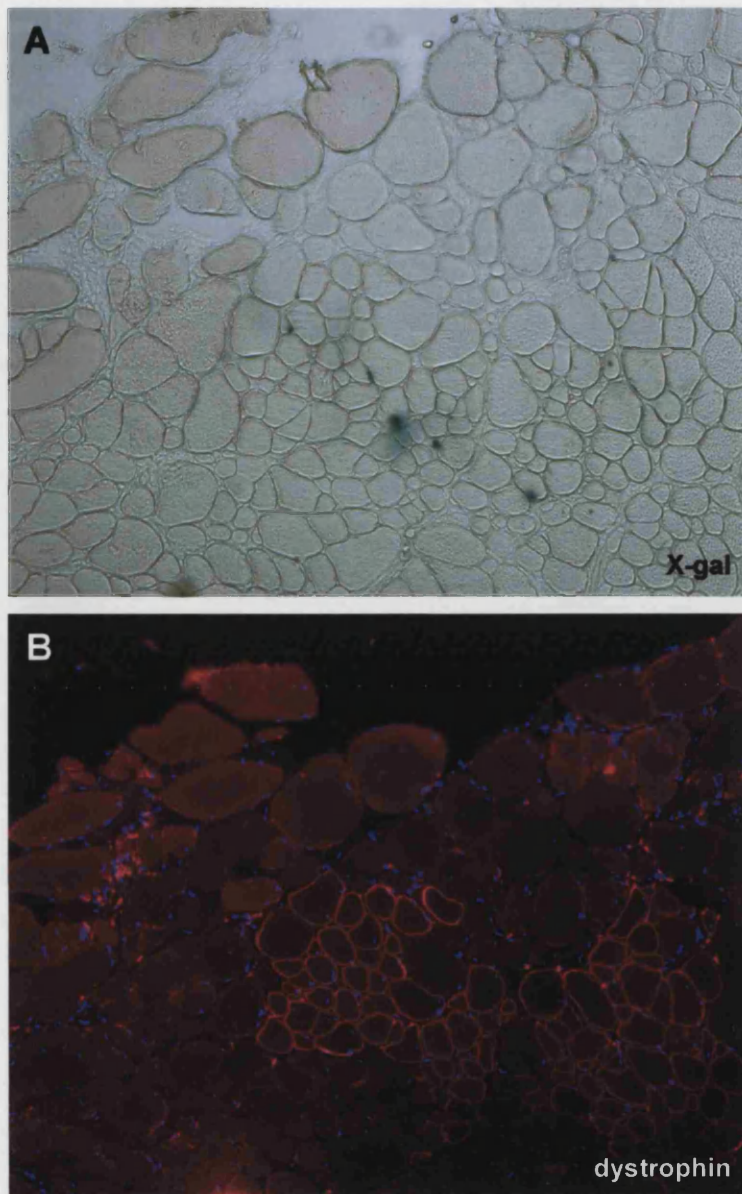


Fig. 3.17. Identification of donor-derived muscle formation in an irradiated *mdx*-nude TA muscle engrafted with a single EDL myofibre and harvested at the 12 week timepoint. Donor-derived muscle is identifiable by X-gal staining to localise β -gal activity (A) and in a serial section, fluorescence immunohistochemistry for dystrophin protein, counterstained with DAPI (B). Original magnification x100.

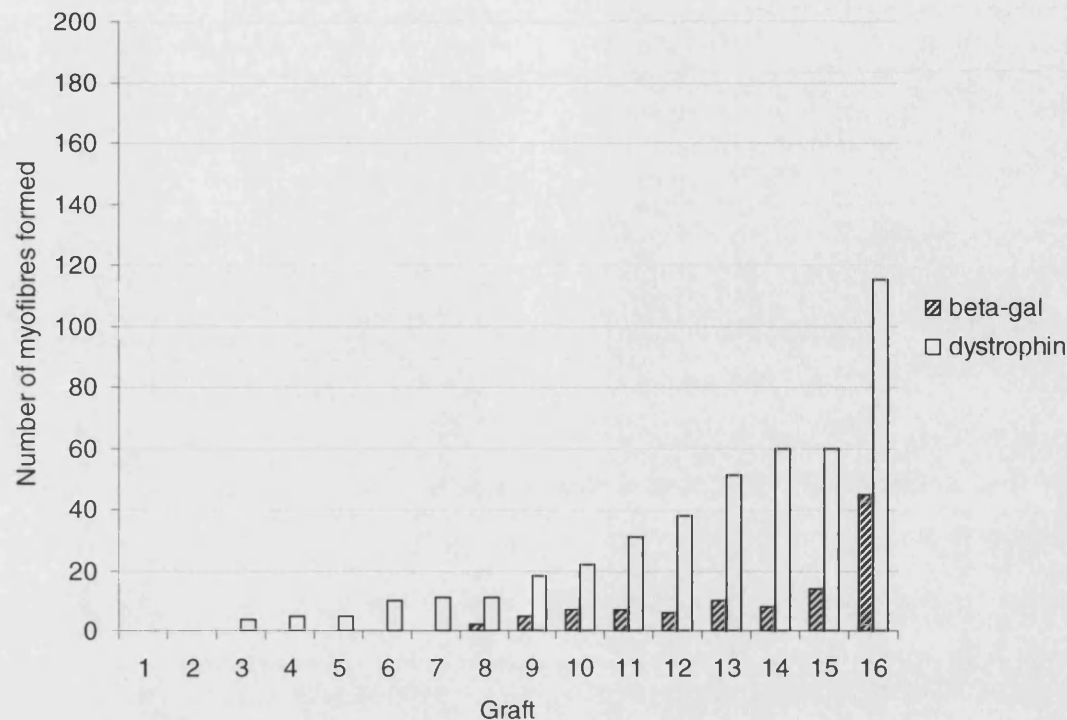


Fig. 3.18. Histogram showing muscle formation by individual grafts of 3F-*nLacZ*-2E EDL single myofibres 12 weeks post-grafting. Ranked in order of the number of dystrophin⁺ myofibres. Counts were made of the maximum numbers of dystrophin⁺ and β -gal⁺ myofibres in single serial sections of engrafted muscles. 9/16 (56%) of engrafted muscles contained both β -gal activity and dystrophin expression.

In total, an estimated 112 EDL satellite cells (based on 16 EDL single myofibres with an average of 7 associated satellite cells) were grafted in this experiment, generating a total of 104 β -gal⁺ myofibres and 406 dystrophin⁺ myofibres. Therefore the absolute efficiency of muscle formation was 0.93 β -gal⁺ myofibres and 3.63 dystrophin⁺ myofibres per grafted satellite cell. If grafts that generated no donor-derived muscle are excluded from the calculation, the efficiency was 1.65 β -gal⁺ myofibres and 6.44 dystrophin⁺ myofibres per satellite cell. Similarly, in the single graft that made the most muscle, the estimated efficiency was 6.43 β -gal⁺ myofibres and 16.43 dystrophin⁺ myofibres per satellite cell.

	3 week timepoint	12 week timepoint
No. of grafts	34	16
Percentage of successful ^a grafts	32%	56%
Mean β -gal ⁺ myofibres formed by successful grafts	27.1 (\pm 37.2)	11.6 (\pm 13.0)
Mean dystrophin ⁺ myofibres formed by successful grafts	47.8 (\pm 59.4)	46.3 (\pm 31.7)
Overall efficiency ^b	2.21	3.63

^a Grafts with ≥ 1 β -gal⁺ nucleus. ^b Efficiency= the total donor-derived myofibres formed divided by the total myogenic cells grafted.

Table 3.8. Comparison of the quantities of muscle formed by grafts of single myofibres derived from the EDL muscles of 42 to 49-day-old 3F-*nLacZ*-2E mice, when harvested after 3 weeks or 12 weeks.

In comparison with EDL myofibre grafts harvested at the 3 week timepoint, grafts harvested at the 12 week timepoint generated muscle more frequently, but in similar average quantity (**Table 3.8**). This implies that the increase in overall efficiency is attributable to an increase in the number of the number of successfully-engrafted muscles, rather than expansion of existing clusters. However, the comparative distributions of the numbers of donor-derived myofibres formed by individual grafts at the different timepoints argues against this. Only 4/34 (12%) of engrafted muscles harvested at the 3 week timepoint generated >30 dystrophin⁺ myofibres, whereas 6/16 (38%) of engrafted muscles harvested at the 12 week timepoint contained >30 dystrophin⁺ myofibres (**Figs. 3.11 & 3.17**). The single large outlier at the 3 week timepoint makes a very large contribution to the mean amount of muscle formed; in the absence of the outlier, there would also be an increase over time in the mean number of myofibres formed by successful grafts (**Figs. 3.11 & 3.17**).

Whilst at the 3 week timepoint, 57% of dystrophin⁺ myofibres contained β -gal activity, at the 12 week timepoint, only 25% of dystrophin⁺ myofibres contained β -gal activity (**Table 3.8**). This implies that expression of the *nLacZ* transgene declines over time. The 7/16 engrafted muscles that did not contain β -gal activity contained 5.7 (\pm 4.3) dystrophin⁺ myofibres, presumably revertants. The number of revertant myofibres was unexpectedly low for irradiated TA muscles in animals of this age (Q.

Lu, personal communication). The effect of the nude background on revertant myofibre formation has not, however, been specifically studied, and speculatively could have been a determining factor.

The results of this experiment suggest that muscle formation by single myofibre grafts is spontaneously augmented over time.

3.5 Grafts of single myofibres derived from juvenile muscles

3.51 Aim

In previous experiments, single myofibre grafts were derived from the relatively mature muscles of young adult mice (3.4). The objective of this study was to investigate the myogenic potential of single myofibres derived from juvenile, growing muscles.

3.52 The myogenic potential of single myofibres derived from juvenile muscles

Single myofibres were isolated (2.21) from the EDL muscles of a 19-day-old male 3F-*nLacZ*-2E mouse (2.13), and from the EDL and TA muscles of a 26-day-old male 3F-*nLacZ*-2E mouse (2.13). Single myofibre grafts were inserted into 25 pre-irradiated TA muscles of *mdx*-nude host mice (2.53, 2.55) (EDL myofibres from 19 d donor n=9; EDL myofibres from 26 d donor n=12; TA myofibres from 26 d donor n=4). Engrafted TA muscles were removed after 3 weeks, and three serial 7 μ m sections taken at 100 μ m intervals throughout each entire muscle body (2.61, 2.62). In the first set of sections X-gal staining was used to localise β -gal activity (2.77), and in the second set, dystrophin protein was detected immunohistochemically (2.76). For each engrafted muscle, counts were made of the maximum number of β -gal⁺ and dystrophin⁺ myofibres present in one section from the same level (Fig. 3.19). 4/9 (44%) of muscles engrafted with EDL myofibres derived from the 19-day-old donor generated 26.0 (\pm 32.7) β -gal⁺ myofibres and 28.0 (\pm 35.3) dystrophin⁺ myofibres. 10/12 (83%) of muscles engrafted with EDL myofibres derived from the 26-day-old donor generated 11.6 (\pm 9.3) β -gal⁺ myofibres and 38.3 (\pm 27.9) dystrophin⁺ myofibres (Fig 3.19 & Fig. 3.20, Table 3.9).

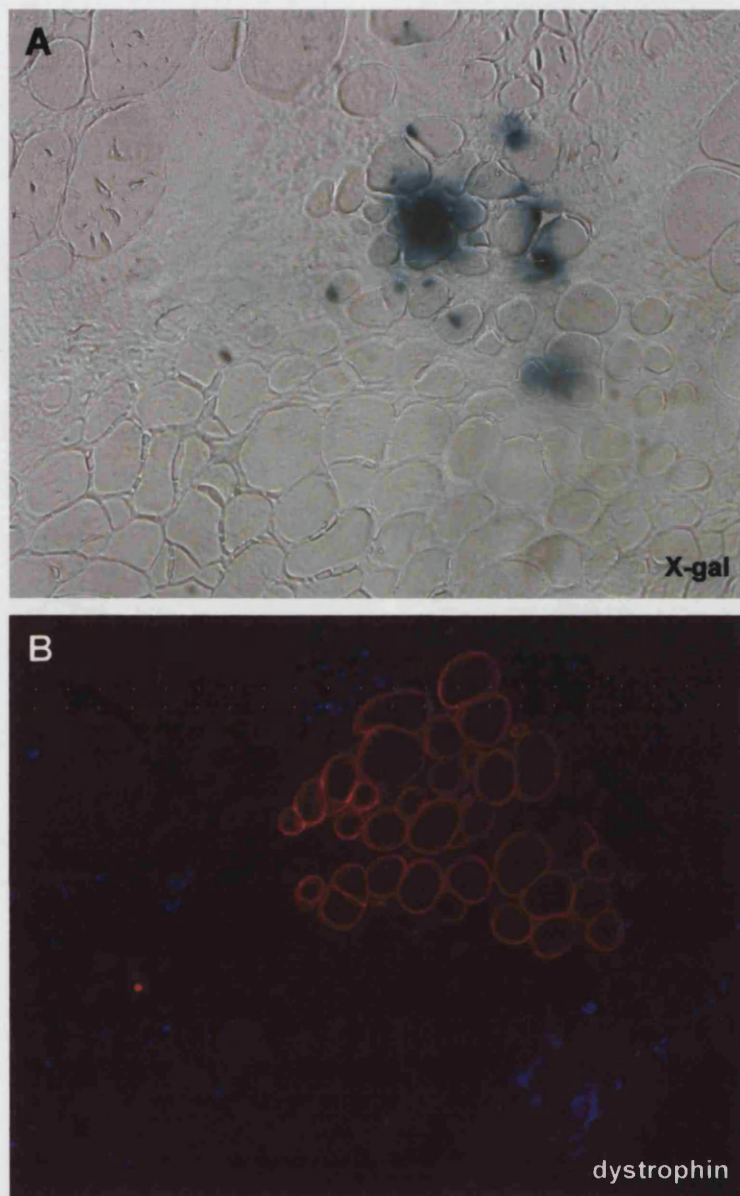


Fig. 3.19. Identification of donor-derived muscle formation in an irradiated *mdx*-nude TA muscle engrafted with a single myofibre isolated from the EDL muscle of a 26-day-old juvenile 3F-*nLacZ*-2E mouse. Donor-derived muscle is identifiable by X-gal staining to localise β -gal activity (A) and in a serial section, fluorescence immunohistochemistry for dystrophin protein, counterstained with DAPI (B). 3 week timepoint. Original magnification x200.

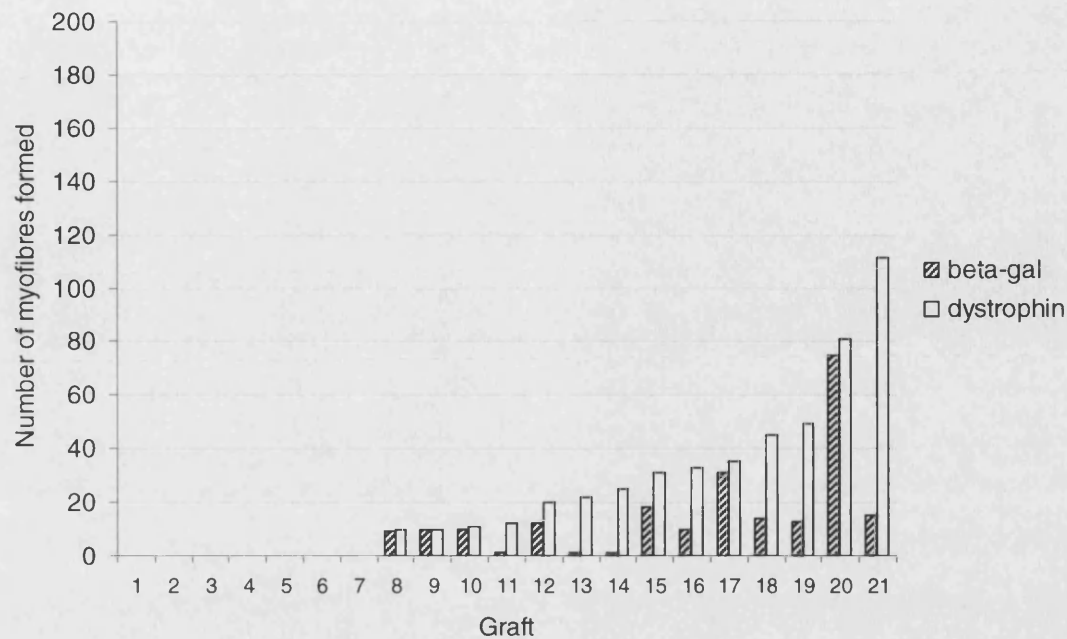


Fig. 3.20. Histogram showing muscle formation by individual grafts of single myofibres derived from the EDL muscles of 19 to 26-day-old 3F-*nLacZ*-2E mice. Ranked in order of the number of dystrophin⁺ myofibres. 3 weeks after grafting, counts were made of the maximum numbers of dystrophin⁺ and β -gal⁺ myofibres in single serial sections of engrafted muscles. 14/21 (67%) of engrafted muscles contained both β -gal activity and dystrophin expression. Engrafted muscles with no detectable β -gal activity were not stained for dystrophin. Pooled data from two experiments.

	Adult 42 to 49-day-old donors (n=4)	Juvenile 19 to 26-day-old donors (n=2)
No. of grafts	34	21
Percentage of successful ^a grafts	32%	67%
Mean β -gal ⁺ myofibres formed by successful grafts	27.1 (\pm 37.2)	15.7 (\pm 18.8)
Mean dystrophin ⁺ myofibres formed by successful grafts	47.8 (\pm 59.4)	35.4 (\pm 29.2)
Overall efficiency ^b	2.21	3.37

^a Grafts with ≥ 1 β -gal⁺ nucleus. ^b Efficiency= the total donor-derived myofibres formed divided by the total myogenic cells grafted.

Table 3.9. Comparison of the quantities of muscle formed by grafts of single myofibres derived from the EDL muscles of 42 to 49-day-old and 19 to 26-day-old 3F-*nLacZ*-2E mice.

Overall, grafts of EDL myofibres derived from juvenile donors generated muscle more than twice as frequently as grafts of myofibres derived from adult donors, although the mean quantity of muscle formed by each successful graft was slightly less. The overall increase in efficiency is therefore attributable to increased frequency of muscle formation rather than the generation of larger clusters of myofibres (**Table 3.9**). 3/4 (75%) of muscles engrafted with TA myofibres derived from the 26-day-old donor generated 9.7 (\pm 7.4) β -gal⁺ myofibres and 34.7 (\pm 22.4) dystrophin⁺ myofibres (**Fig. 3.21**). One individual graft generated 19 β -gal⁺ myofibres and 66 dystrophin⁺ myofibres. Of 42 grafts derived from the TA muscles of adult mice no graft generated more than 11 β -gal⁺ myofibres (**2.45**).

The results of this study show that the discrete populations of satellite cells associated with single myofibres derived from juvenile muscles have greater myogenic potential than those with single myofibres derived from adult muscles.

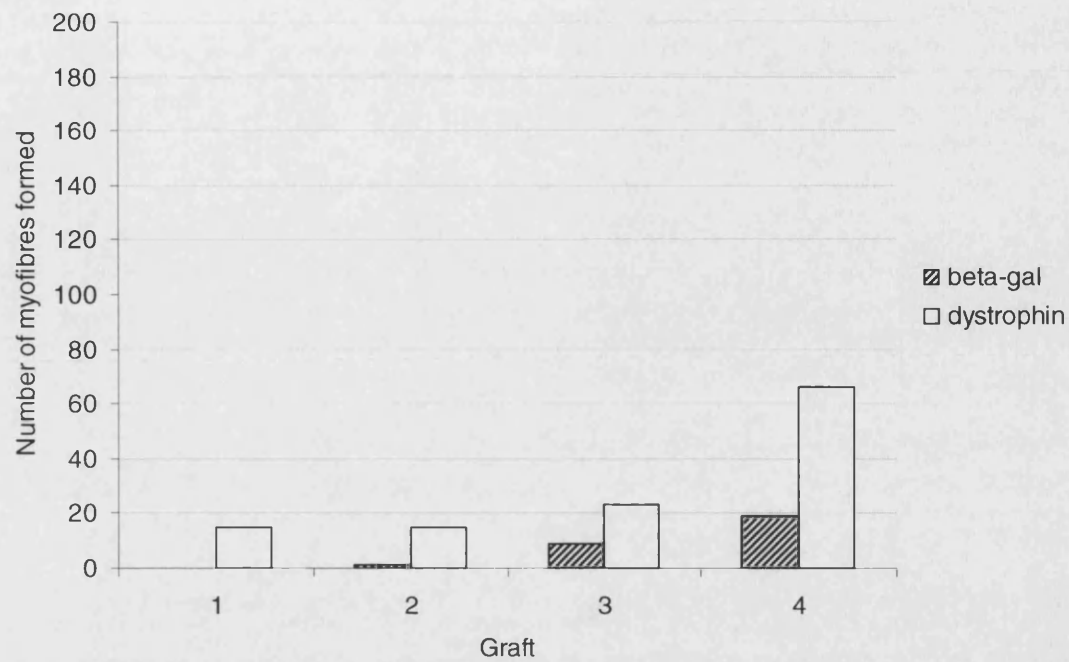


Fig. 3.21. Histogram showing muscle formation by individual grafts of single myofibres derived from the TA muscles of a 26-day-old 3F-*nLacZ*-2E mouse. Ranked in order of the number of dystrophin⁺ myofibres. 3 weeks after grafting, counts were made of the maximum numbers of dystrophin⁺ and β -gal⁺ myofibres in single serial sections of engrafted muscles. 3/4 (75%) of engrafted muscles contained both β -gal activity and dystrophin expression.

3.6 Investigation of the radiation-sensitivity of satellite cells

3.61 Aim

Skeletal muscle harbours a population of radiation-resistant stem cell-like cells which are the origin of efficient muscle regeneration after acute damage (Heslop *et al.* 2000). The results of experiments previously described in this chapter indicate that satellite cells have a stem cell-like myogenic potential. The objective of this study was to determine whether the stem cell-like satellite cells in single myofibre grafts are derived from the stem cell-like radiation-resistant population.

3.62 Cells emanating from irradiated single myofibres in culture

Two 44-day-old 3F-*nLacZ*-2E mice (2.13) were anaesthetised (2.52) and the right hindlegs exposed to 18 Gy γ -irradiation (2.53). The contralateral left hindlegs functioned as non-irradiated controls. 3 d post-irradiation, single myofibres were prepared from the EDL and soleus muscles (2.21) of irradiated and non-irradiated hindlegs. Myofibres from the two animals were pooled. Single myofibres from the same muscles were used for both tissue culture (3.62) and grafting (3.63) experiments. Since little is known about the timecourse of radiation effects on satellite cells, the 3 d timepoint was selected since it has been used successfully to ablate the satellite cell compartment of host mice prior to grafting (J. Morgan, personal communication, Gross *et al.* 1999)

The effect of irradiation on the proliferative competence of satellite cells was investigated. 24 single myofibres isolated from control non-irradiated EDL muscles and 24 single myofibres isolated from irradiated EDL muscles were plated in the separate wells of 24 well plates, and maintained as adherent cultures (2.21). After 72 h, the number of cells emanating from each myofibre was counted. Non-irradiated control single myofibres (n=23) gave rise to 46.0 (\pm 22.0) cells, and irradiated single myofibres (n=24) gave rise to 2.1 (\pm 2.3) cells (Fig. 3.22), a statistically significant difference (Mann-Whitney, $p > 0.05$). The majority of wild-type single myofibre cultures give rise to myogenic satellite-derived cells only, but about 1/50 are contaminated by non-muscle cell types, probably fibroblasts (Rosenblatt *et al.* 1995, Bockhold *et al.* 1998). In this experiment, the myogenic lineage of emanating cells

was confirmed by morphological observation only; non-myogenic cells might therefore have made a minor contribution to counts.

During the 72 h time-course, satellite cells in control cultures migrated away from myofibres and proliferated. In cultures derived from irradiated muscles, satellite cells migrated from myofibres, and sometimes appeared to undergo one cell division before detaching from the plate and presumably dying. These observations show that 18 Gy irradiation is sufficient to ablate the replication-competence of EDL muscle satellite cells when subsequently placed into tissue culture. This finding is supported by the results of another study which demonstrated a similar result using single myofibre explants derived from 18 Gy-irradiated TA muscles (Heslop *et al.* 2000).

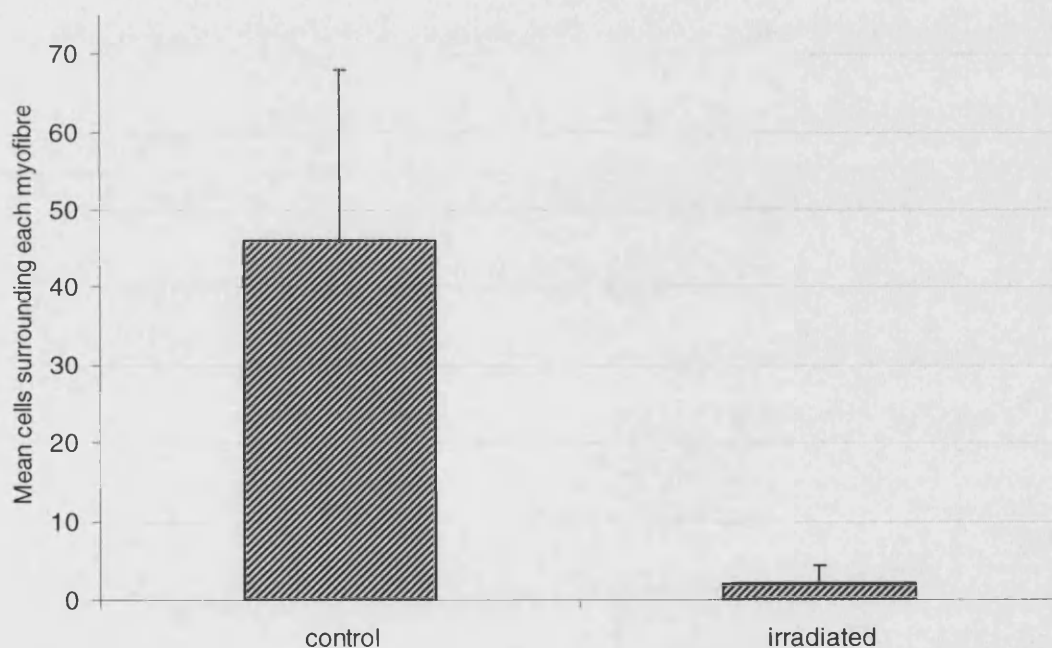


Fig. 3.22. Bar graph showing the numbers of cells surrounding myofibres derived from control and 18 Gy irradiated EDL muscles. EDL muscles were obtained from two 3F-*nLacZ*-2E mice where the right hindleg had been exposed to 18 Gy irradiation 3 days previously, and the left contralateral hindleg functioning as a non-irradiated control. Myofibres were maintained as adherent cultures and counts made 72 h after plating. These data show that whilst a few cells emanate from myofibres derived from irradiated muscles, they do not have proliferative potential.

3.63 The myogenic potential of irradiated EDL single myofibres

Single myofibres isolated from irradiated EDL (n=10) and soleus (n=10) 3F-*nLacZ*-2E muscles were grafted into the pre-irradiated TA muscles of anaesthetised *mdx*-nude mice (2.52, 2.53, 2.55). Engrafted muscles were removed after 3 weeks and processed for immunohistochemistry (2.61). 3 serial 7 µm sections were collected at 100 µm intervals throughout the entire TA muscle body (2.62). Staining of the first set of sections in X-gal solution (2.77) failed to detect β-gal activity in any of the 20 engrafted muscles (Table 3.10). These data demonstrate that similarly to the satellite cells which generate proliferation-competent progeny in tissue culture, the satellite cells responsible for the myogenic activity of single myofibre grafts *in vivo* are sensitive to 18 Gy irradiation.

Origin of single myofibre graft	No. of grafts	Engrafted muscles with ≥1 β-gal ⁺ nuclei
Irradiated 3F- <i>nLacZ</i> -2E EDL muscles	10	0
Irradiated 3F- <i>nLacZ</i> -2E soleus muscles	10	0

Table 3.10. Absence of muscle formation in host TA muscles engrafted with myofibres derived from 18 Gy-irradiated 3F-*nLacZ*-2E muscles.

3.64 The myogenic potential of irradiated and non-irradiated single myofibres in notexin-damaged sites

The myotoxic snake venom, notexin, causes necrosis of skeletal muscle tissue (reviewed, Harris 2003) and has been shown to augment the amount of muscle formed by grafted MPC (Kinoshita *et al.* 1994, Skuk *et al.* 1999, Pye *et al.* 2004). Heslop *et al.* (2000) describe a population of tissue-resident MPC that is only myogenic in response to notexin-induced damage. The purpose of this experiment was to determine whether single myofibre grafts derived from irradiated muscles retain myogenic potential in response to acute damage, or whether their entire myogenic potential is ablated.

As previously, a 43-day-old male 3F-*nLacZ*-2E mouse was anaesthetised (2.52) and the right hindleg exposed to 18 Gy γ -irradiation (2.53). The contralateral left leg served the non-irradiated control. 3 days post-irradiation, single myofibres were isolated from irradiated and non-irradiated EDL muscles (2.21). The hindlegs of 6 *mdx*-nude mice were irradiated (2.53) 3 d prior to grafting. 5 μ l of notexin (10 μ g/ml in PBS) was injected into each host TA muscle (2.56), and a single myofibre immediately grafted into the same site (2.55). Right TA muscles (n=6) received grafts of single myofibres isolated from the irradiated donor EDL muscle, and left TA muscles (n=6) received grafts of single myofibres isolated from the non-irradiated donor EDL muscle.

Engrafted muscles were removed after 3 weeks and processed for immunohistochemistry (2.61). 3 serial 7 μ m sections were collected at 100 μ m intervals throughout the entire TA muscle body (2.62). In the first set of sections X-gal staining was used to localise β -gal activity (2.77), and in the second set dystrophin expression was detected immunohistochemically (2.76). No β -gal expression was detected in any notexin-injected muscles engrafted with irradiated EDL myofibres. 3/6 (50%) of notexin-injected muscles engrafted with non-irradiated EDL myofibres contained 14.0 (\pm 17.4) β -gal⁺ myofibres and 30.3 (\pm 13.7) dystrophin⁺ myofibres (Fig. 3.23, Table 3.11).

Origin of single myofibre graft	Notexin damage to host graft site	No. of grafts	Engrafted muscles with \geq 1 β -gal ⁺ nuclei
Irradiated 3F- <i>nLacZ</i> -2E right EDL muscle	yes	6 (right host legs)	0
Contralateral non-irradiated 3F- <i>nLacZ</i> -2E left EDL muscle	yes	6 (contralateral left host legs)	3

Table 3.11. Muscle formation by myofibres isolated from non-irradiated and contralateral irradiated 3F-*nLacZ*-2E EDL muscles, and grafted into notexin-treated host muscles.

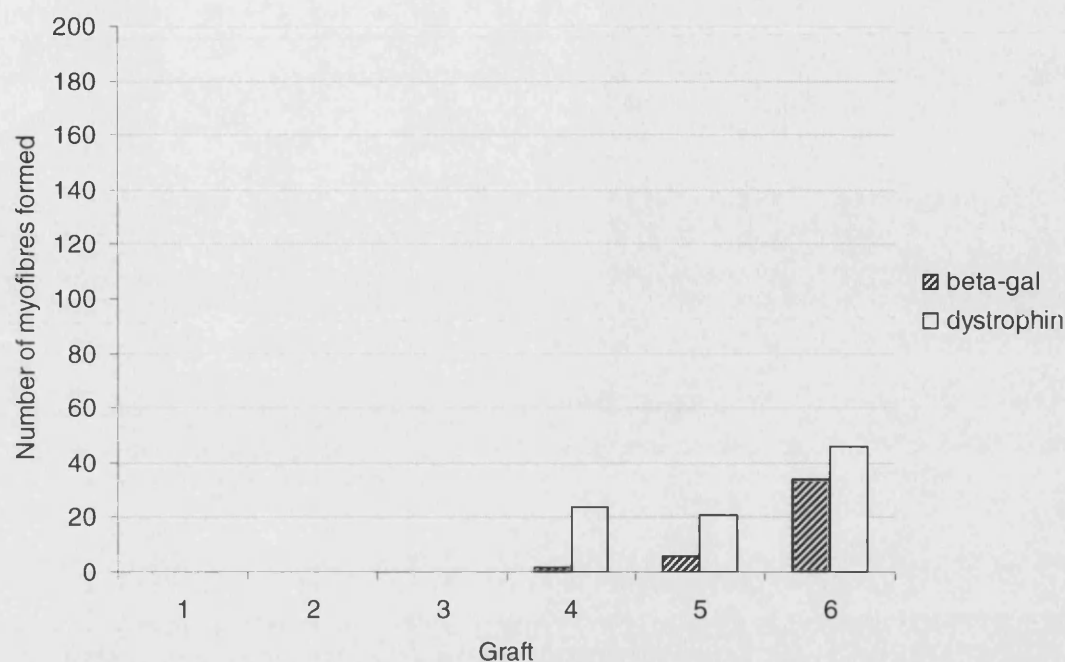


Fig 3.23. Histogram showing muscle formation by individual grafts of single myofibres derived from the non-irradiated EDL muscle of a 42-day-old 3F-*nLacZ*-2E mouse, with notexin injected into the recipient muscle at the time of grafting. Ranked in order of the number of dystrophin⁺ myofibres. 3 weeks after grafting, counts were made of the maximum numbers of dystrophin⁺ and β -gal⁺ myofibres in single serial sections of engrafted muscles. 3/6 (50%) of engrafted muscles contained both β -gal activity and dystrophin expression. Engrafted muscles with no detectable β -gal activity were not stained for dystrophin. 0/6 grafts derived from the contralateral irradiated EDL muscle of the same donor mouse generated β -gal activity (data not shown here).

These data suggest that single myofibre grafts do not harbour radiation-resistant myogenic cells which can be activated in response to acute damage. In total, an estimated 110 irradiated EDL satellite cells (based on 16 single myofibres each with an average of 7 associated satellite cells), and 300 irradiated soleus satellite cells (based on 10 single myofibres each with an average of 30 associated satellite cells), were grafted in these experiments, of which approximately 42 (6 EDL single myofibres) were grafted with notexin. None of these grafts resulted in muscle formation, demonstrating that a radiation-sensitive phenotype is responsible for the myogenic activity of single myofibre grafts. Since the products of grafts were only identifiable when cells had contributed to differentiated muscle, the absence of

proliferation by grafted satellite cells *in vivo* was implied, rather than unequivocally shown. However, since satellite cells derived from irradiated muscles do not proliferate *in vitro* (3.62), lack of proliferation is the most likely explanation for lack of myogenicity *in vivo*.

3.65 The myogenic potential of primary MPC isolated from irradiated muscles

Exposure to of muscles to 18 Gy irradiation ablates the proliferative and myogenic competence of subsequently isolated satellite cells both *in vitro* (Heslop *et al.* 2000, 3.62) and *in vivo* (3.63, 3.64). However, 18 Gy irradiated muscles retain the ability to regenerate after severe injury (Heslop *et al.* 2000). Hypothetically, regeneration could be derived from radiation-resistant cells that are resident committed myogenic progenitors, or alternatively from cells of a non-myogenic lineage which are recruited into a myogenic program in response to damage. This experiment aimed to investigate the myogenic potential of cells isolated from muscles that had been exposed to 18 Gy irradiation, but not otherwise stimulated by damage.

The hindlegs of six 42-day-old 3F-*nLacZ*-2E mice (3 males and 3 females) were exposed to 18 Gy γ -irradiation (2.53). Three days post-irradiation, mice were sacrificed and the EDL, soleus and TA muscles dissected. Pooled muscle groups were separately minced and enzymatically disaggregated to obtain cell suspensions. >95% of isolated cells were viable as defined by exclusion of Trypan blue dye (2.22). 10 μ l of each cell suspension was spotted onto a silane-coated slide and air-dried at RT. The remaining cells were preserved in aliquots under liquid nitrogen. The percentage of desmin⁺ cells in each isolate was determined by immunocytochemistry of air-dried cells (2.74). The numbers of desmin⁺ cells in preparations derived from the different irradiated muscle groups are shown in **Table 3.12**.

	EDL	soleus	TA
Number of muscles	12	12	12
Mean weight (mg)	4.16	5.80	27.50
Viable cells per mg tissue	7.5×10^3	1.44×10^4	8.45×10^3
Desmin ⁺ cells (% of total)	10.27%	30.79%	56.16%
Desmin ⁺ cells per mg tissue	7.70×10^2	4.43×10^3	4.74×10^3
Mean desmin ⁺ cells per muscle	3.2×10^3	2.57×10^4	1.3×10^5

Table 3.12. Isolation of putative myogenic cells from muscles of 42-day-old 3F-*nLacZ*-2E mice that had been exposed to 18 Gy γ -irradiation 3 days previously.

In comparison to primary cell isolates derived from non-irradiated 3F-*nLacZ*-2E muscles (3.31), there were relatively small differences in numbers of viable desmin⁺ cells isolated from irradiated soleus and TA muscles (Tables 3.1 and 3.12). However, very few desmin⁺ cells were obtained from irradiated EDL muscles (Table 3.12). The low yield may be attributable to the technical difficulty of disaggregating very small muscles, or may imply that the cell populations of the EDL muscle have an increased radiation-sensitivity over those of soleus and TA muscles.

The myogenic potential of cells derived from irradiated soleus and TA muscles was investigated by grafting. Immediately prior to grafting, cells were thawed, washed in growth medium and cell suspensions prepared such that 5×10^4 desmin⁺ cells were contained per 4 μ l medium. Thus as previously (3.31), the total numbers of cells in each graft differed slightly between the two isolates, but the number of desmin⁺ cells remained constant. 4 μ l aliquots of cell suspension were grafted into the pre-irradiated TA muscles of four anaesthetised *mdx*-nude mice (2.52, 2.53, 2.55). Cells derived from irradiated soleus muscles were grafted into right host TA muscles and cells derived from irradiated TA muscles were grafted into contralateral left host TA muscles. Engrafted muscles were removed after 3 weeks and processed for immunohistochemistry (2.61). 3 serial sections were collected at 100 μ m intervals

throughout 2/3 of the TA muscle body (2.62). In the first set of sections, β -gal activity was localised using X-gal staining (2.77). No muscles engrafted with irradiated TA cells contained identifiable β -gal activity. Whilst 3/4 muscles engrafted with irradiated soleus cells contained no β -gal activity, one engrafted muscle contained a small group of 5 β -gal⁺ myofibres (Fig. 3.24, Table 3.13).

Origin of primary cell isolate	No. of grafts	Engrafted muscles with ≥ 1 β -gal ⁺ nuclei
Irradiated 3F- <i>nLacZ</i> -2E soleus muscles	4	1
Irradiated 3F- <i>nLacZ</i> -2E TA muscles	4	0

Table 3.13. Infrequency of muscle formation in host TA muscles engrafted with primary cells derived from 18 Gy-irradiated 3F-*nLacZ*-2E muscles.

These data demonstrate that the myogenic activity of grafts of enzymatically-disaggregated muscle cells (3.32) is almost entirely localised to the radiation-sensitive compartment of MPC.

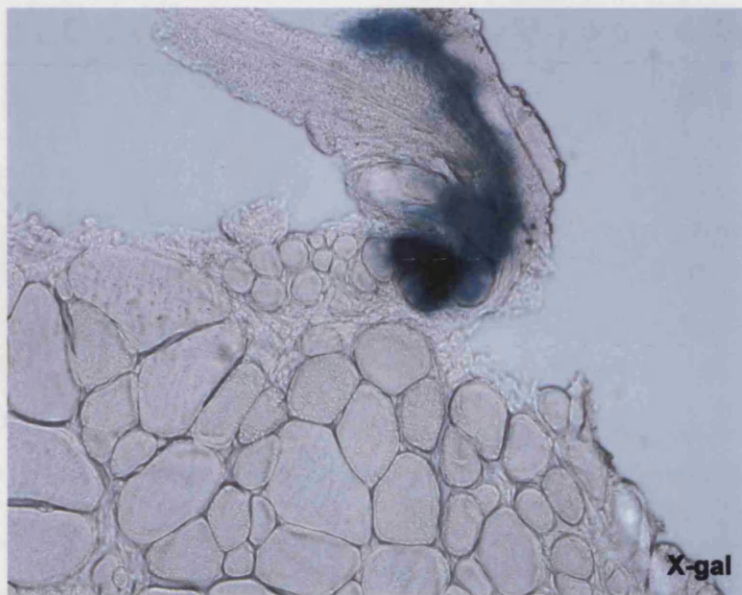


Fig. 3.24. Identification of donor-derived muscle formed from a graft of 5×10^4 primary desmin⁺ cells isolated from 18 Gy-irradiated 3F-*nLacZ*-2E soleus muscles. Section of engrafted TA muscle stained in X-gal to localise β-gal activity. The small cluster of β-gal⁺ myofibres shown here was the only donor-derived muscle identified in a total of 8 muscles grafted with 5×10^4 desmin⁺ cells derived from irradiated soleus or TA muscles. 3 week timepoint. Original magnification x200.

3.7 Comments on results

The quantity of muscle generated by grafts of clone SFIK immortalised myoblasts was proportional to the numbers of cells within the graft. However, the efficiency with which muscle was formed declined as the number of myoblasts increased, such that smaller numbers of myoblasts formed proportionately larger amounts of muscle. Grafts of 5×10^4 primary, non-cultured myogenic cells formed muscle with only slightly greater efficiency than grafts of 5×10^4 SFIK immortalised myoblasts. This finding could be interpreted as evidence that extensive *in vitro* expansion does not significantly impair myogenic potential, or alternatively indicate that only significantly-myogenic clones give rise to cell lines.

In grafts of primary muscle cells, the efficiency with which muscle was formed did not differ according to the muscle group (EDL, soleus or TA) from which cells were isolated, implying that the MPC populations of these muscles have similar regenerative potential (**Fig. 3.7, Table 3.6**). The low efficiency of muscle formation by all MPC grafts is in agreement with data generated by many previous studies which have failed to demonstrate donor-derived muscle formation greatly exceeding parity (reviewed, Partridge 2003). The number of desmin⁺ cells obtained per muscle in preparations of enzymatically disaggregated cells (**Table 3.1**) was unexpectedly high, exceeding the predicted maximum number of satellite cells present. For instance, the murine soleus muscle contains approximately 900 myofibres (personal communication, P. Zammit), each with an average of 22 satellite cells (**3.42**). Therefore the estimated total number of satellite cells per muscle is 1.98×10^4 . However, a mean of 4.37×10^4 desmin⁺ cells were obtained per muscle. These data predict that uninjured adult skeletal muscles harbour substantial numbers of desmin⁺ (presumptively myogenic) cells that reside outside the satellite cell compartment.

Grafts of small discrete populations of satellite cells maintained in association with a viable parent myofibre were found, in some cases, to generate over 100 new myofibres within the 3 week timecourse. In comparison with primary myoblasts isolated from equivalent muscles and grafted into equivalent host environments, single myofibre grafts formed muscle with several thousand times greater efficiency (**Table 3.6**). This finding demonstrates that the considerable proliferative potential of

at least a subset of satellite cells is critically dependent on their endogenous association with their parent myofibre. Importantly, single myofibres grafts inserted into notexin-treated sites still generated proportionately large amounts of muscle, despite the fact that the myofibre itself would have degraded within a few hours (3.64). This implies the existence of an initial, myofibre-dependent activation event which generates highly proliferative myogenic progeny that are not functionally dependent on an interaction with the parent myofibre. Speculatively, this event could be the activation and division of a more primitive, myofibre-dependent stem cell, to generate a daughter cell whose non-myofibre-dependent progeny migrate away and proliferate to form a pool of MPC. The finding that muscle formation by EDL grafts is augmented over time (Table 3.8) shows that an MPC population persists beyond the 3 week timepoint, and further, the increased frequency of muscle formation at 12 weeks over 3 weeks (Table 3.8) suggests that at least a proportion of MPC are generated by activation events that take place some time after grafting.

Whilst EDL and soleus satellite cells generated muscle with relatively similar overall efficiency (Table 3.6), though different frequency (Table 3.5), the efficiency of muscle formation by TA satellite cells was substantially and consistently less than satellite cells from the other two muscles. TA myofibres and EDL myofibres have similar numbers of associated satellite cells (Fig. 3.9) and were found to generate muscle with similar frequency (Table 3.5). Whilst many EDL myofibres generated only small amounts of muscle, a small subset generated large amounts (Figs. 3.10 & 3.11). This highly-myogenic subset was apparently absent from adult-derived TA myofibre grafts (Figs. 3.14 & 3.15), though one individual juvenile-derived TA myofibre graft generated 19 β -gal⁺ myofibres (Fig 3.21). The small number of grafts of juvenile TA myofibres (n=4) cannot justify any firm conclusions; but speculatively, these data could be explained by an endogenous process whereby the proliferative/myogenic potential of TA satellite cells becomes depleted by postnatal growth. The data do not exclude the possibility that adult TA satellite cells have similar myogenic potential to those of EDL and soleus muscles, but proliferate more slowly and consequently formed less muscle at the single timepoint analysed. The finding that TA satellite cells formed less muscle than EDL or soleus satellite cells, in a TA environment, directly contradicts the findings of other workers who have suggested that fibre-type compatibility is a positive determinant of myogenic potential (Qu &

Huard 2000). These data show that satellite cells have qualities that are distinct to their muscle of origin, and that persist outside that environment.

Muscle formation by EDL myofibres isolated from the muscles of juvenile donors occurred with about twice the frequency, and with greater overall efficiency, of that by EDL myofibres from adult donors (**Table 3.9**). Since the numbers of satellite cells associated with myofibres from juvenile muscles were not quantified, this difference could be attributable to either greater numbers of cells, an increased proportion of endogenously-activated cells, or differences in proliferative rate. Nevertheless, these data suggest that the activation state of the donor muscle may be a factor which determines the efficiency of donor-derived muscle formation in the engrafted muscle.

Myofibres isolated from 18 Gy-irradiated donor muscles did not form any identifiable donor-derived products in engrafted muscles, either with or without notexin-induced damage (**3.63, 3.64**). Since the total number of grafted satellite cells was relatively small, the data do not absolutely exclude the possibility that radiation-resistant cells exist within the satellite cell compartment, but at a frequency too low to be detectable within the context of these experiments. Irradiated muscles were enzymatically disaggregated to obtain a larger sample of cells. However, grafts of 5×10^4 desmin⁺ cells derived from irradiated muscles generated a very small amount of muscle in only 1/8 cases (**Table 3.13, Fig. 3.24**). Together, these data show that the cells which generate muscle after grafting are radiation sensitive both within single myofibre grafts and grafts of enzymatically disaggregated cells. Hypothetically, radiation-resistant cells might have been present in grafts of enzymatically-disaggregated cells (**3.65**), but remained non-myogenic in the absence of acute damage. Alternatively, the cells responsible for endogenous regeneration in irradiated muscles (Heslop *et al.* 2000) could be recruited from the circulation or from other resident lineages during acute regenerative events.

In summary, the results of the experiments described in this chapter demonstrate the existence of a satellite cell population which has a stem cell-like myogenic potential, is radiation-sensitive and exhibits distinct inter-muscle variability.

Chapter 4

The potential of satellite cells to self-renew

4.1 Background and aim of study

4.1.1 Background to work

Whilst a progenitor cell may undergo extensive proliferation, it does not have the ability to generate another cell like itself, and once its progeny have differentiated, the complete regenerative potential of the original cell is fulfilled. The ability to self-renew is a defining quality of stem cells, and one which allows them to repeatedly generate progeny with the ability to form new differentiated tissue, thereby maintaining a sustainable regenerative source (Lajtha 1979, Moore 1979, Morrison *et al.* 1997, Anderson *et al.* 2001, Elwood 2004).

Satellite cell numbers are maintained throughout juvenile growth (Schultz 1996) and a limited number of adult regenerative events (Schultz 1984, Morlet *et al.* 1989, Reimann *et al.* 2000), but decline in both absolute numbers and proliferative competency with age (Gibson & Schultz 1983, Sadeh 1988, Bockhold *et al.* 1998, Di Donna *et al.* 2000, Kadi *et al.* 2004, Sajko *et al.* 2004). Whilst there is therefore strong evidence that the adult satellite cell pool has the potential for postnatal renewal, albeit finite, the origin of postnatally-renewed satellite cells has not been unequivocally established (**Fig 1.2**). Self-renewal has been postulated as the mechanism by which the satellite cell compartment is maintained (reviewed, Zammit & Beauchamp 2004). The results of *in vitro* experiments have shown that transformed C2C12 cells (Yoshida *et al.* 1998), primary human myoblasts (Barroffio *et al.* 1996) and mouse satellite cells (Zammit *et al.* 2004) generate undifferentiated reserve populations when the majority population differentiate, phenomena which predict an endogenous pattern of self-renewal.

In vivo, grafts of cloned immortalised myoblasts and primary neonatal myoblasts give rise to satellite cells that are proliferation-competent *in vitro* upon re-isolation in

single myofibre preparations (Blaveri *et al.* 1998, Heslop *et al.* 2001, Cousins *et al.* 2004). In addition, immortalised myoblasts can be serially passaged through several host animals with intervening periods of *in vitro* expansion (Morgan *et al.* 1994). However, graft-derived reserve cells have a somewhat limited ability to participate in damage-induced regeneration *in vivo* (Gross & Morgan 1999), suggesting that *in vitro* replicative competence does not necessarily predict proliferative and myogenic potential *in vivo*. Since all the above studies used grafts of large numbers (5×10^5) of cells, it was not possible to determine whether donor-derived satellite cells were generated stochastically from cells without significant *in vivo* myogenic potential, or whether they were the product of a clonogenic cell which originally generated both differentiation-committed and reserve progeny. In addition, it is not known whether donor-derived satellite cells express appropriate phenotypic markers. One study identified expression of Myf5- β -gal fusion protein in satellite cells generated from grafts of primary neonatal myoblasts derived from the *Myf5^{nlacZ/+}* mouse (Heslop *et al.* 2001); however, since the transplanted population consisted of a mixture of different phenotypes, the precise cellular origin of the new satellite cells is ambiguous. In addition, the use of cells of neonatal origin could not exclude the possibility that new satellite cells were generated by a phenotype which does not persist into adulthood. In the absence of *in vitro* expansion, the ability of defined satellite cells to generate new defined satellite cells has not been formally demonstrated.

4.12 Aims of work

This experiments described in this chapter aimed to determine the potential of adult satellite cells to self-renew their own population by generating new anatomically- and phenotypically-defined satellite cells, and to investigate the frequency with which new satellite cell formation occurs.

4.13 Note on genetic markers of graft-derived satellite cells

Expression of Myf5- β -gal fusion protein has been established as a marker of quiescent satellite cells in the *Myf5^{nLacZ/+}* mouse (Beauchamp *et al.* 2000, Heslop *et al.* 2001, Zammit *et al.* 2002) (**Fig. 4.1**). Myf5- β -gal fusion protein is expressed by the nuclei of immature myofibres during postnatal development, but in the adult, is lost in the nuclei of mature myofibres (Tajbakhsh *et al.* 1995) and retained only by satellite cells (Beauchamp *et al.* 2000). The *Myf5^{nLacZ/+}* mouse is therefore ideally suited as a genetically-marked donor strain for investigation of satellite cell formation after grafting.

Since Myf5- β -gal fusion protein is expressed by both replication-competent cells and newly-formed myotubes, in the absence of anatomical markers *in vitro* its expression does not discriminate between differentiated and undifferentiated nuclei. In tissue culture experiments, Pax7 was used as a marker of undifferentiated cells since in the mouse, its expression is lost after commitment to differentiation (Zammit *et al.* 2004a).

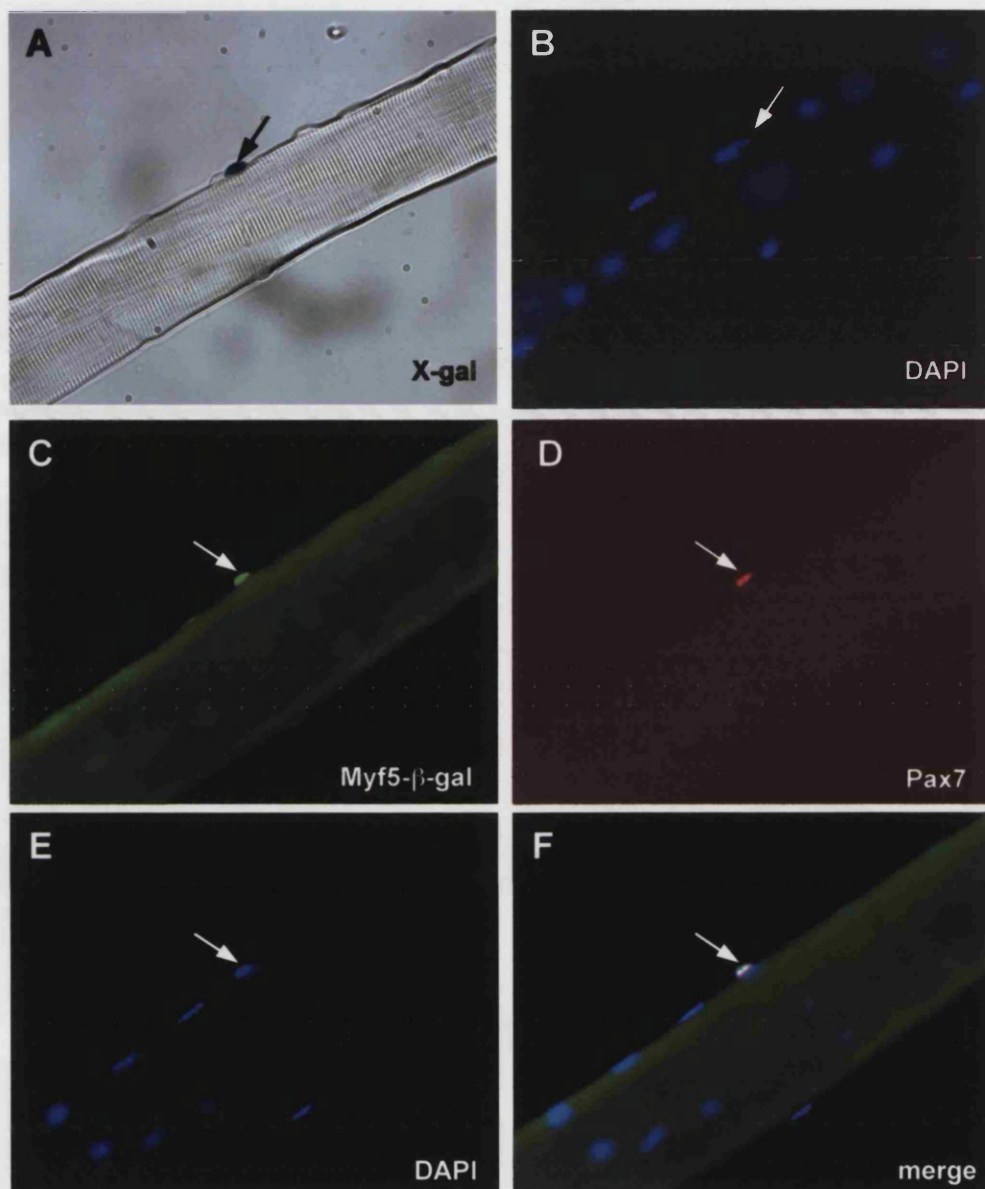


Fig. 4.1. Identification of satellite cells associated with single myofibres isolated from the EDL muscles of a *Myf5^{nLacZ/+}* mouse. Incubation in X-gal followed by DAPI staining (A-B) localises β -gal activity to a satellite cell nucleus (arrowed), which does not translocate to adjacent myonuclei identifiable only by DAPI staining. In a different myofibre (C-F) satellite cells are identified by immunocytochemistry for β -gal and Pax7. A satellite cell nucleus (arrowed) co-expresses Myf5- β -gal and Pax7 proteins. Myonuclei express neither of these markers, but are identified by DAPI staining. Original magnification x400.

4.2 The ability of a satellite cell-derived clone to generate new satellite cells

4.21 Aim

The objective of this study was to investigate the ability of a conditionally-immortalised cell line to give rise to anatomically-defined satellite cells which express appropriate phenotypic markers.

4.22 Preparation of a *Myf5^{tsA58}* satellite cell-derived cell line

A cell line expressing *Myf5^{tsA58}* was generated. Single myofibres were isolated (2.21) from the EDL muscles of a 42-day-old male *H-2K^b-tsA58/ Myf5^{tsA58/+}* double heterozygote mouse (2.16), and cultured individually in the wells of 24-well plates. After 24 h, myofibres were removed and remaining satellite-derived cells expanded under permissive conditions (2.23). Dilution cloning (2.23) was used to obtain six cell lines. Cells from each line were immunostained for expression of desmin and T antigen proteins (2.74) and nuclear β -gal activity localised by staining in X-solution (2.77), to respectively confirm myogenic lineage (Li *et al.* 1994), presence of the immortalising tsA58 antigen, and expression of Myf5- β -gal fusion protein. All cells in all clones were found to both express desmin and tsA58 and to exhibit β -gal activity (data not shown). Fusion assays (2.42) determined that clone Myf5E2 had the highest fusion index (73%). Fluorescence immunocytochemistry was used to survey expression of satellite cell marker proteins in clone Myf5E2 cells (2.74). In proliferating cells maintained under permissive conditions (2.23), nuclear expression of Pax7 and MyoD was almost universal. Immunocytochemistry using an anti- β -gal antibody resulted in a variable pattern of staining, with some cells showing very strong expression of Myf5- β -gal fusion protein, and others staining only weakly. This heterogeneity is consistent with the findings of previous studies which have shown that *Myf5* expression is cell cycle regulated (Kitzmann *et al.* 1998). In differentiated cultures, nuclei within myotubes did not express Pax7, but sometimes expressed myogenin. Cells which remained mononucleate in differentiated cultures variously retained expression of Pax7, expressed myogenin, or were negative for both these markers. Pax7 and myogenin were not co-expressed (Fig. 4.2). These staining patterns suggest that conditional immortalisation does not significantly perturb normal patterns of MRF and Pax7 expression in Myf5E2 cells.

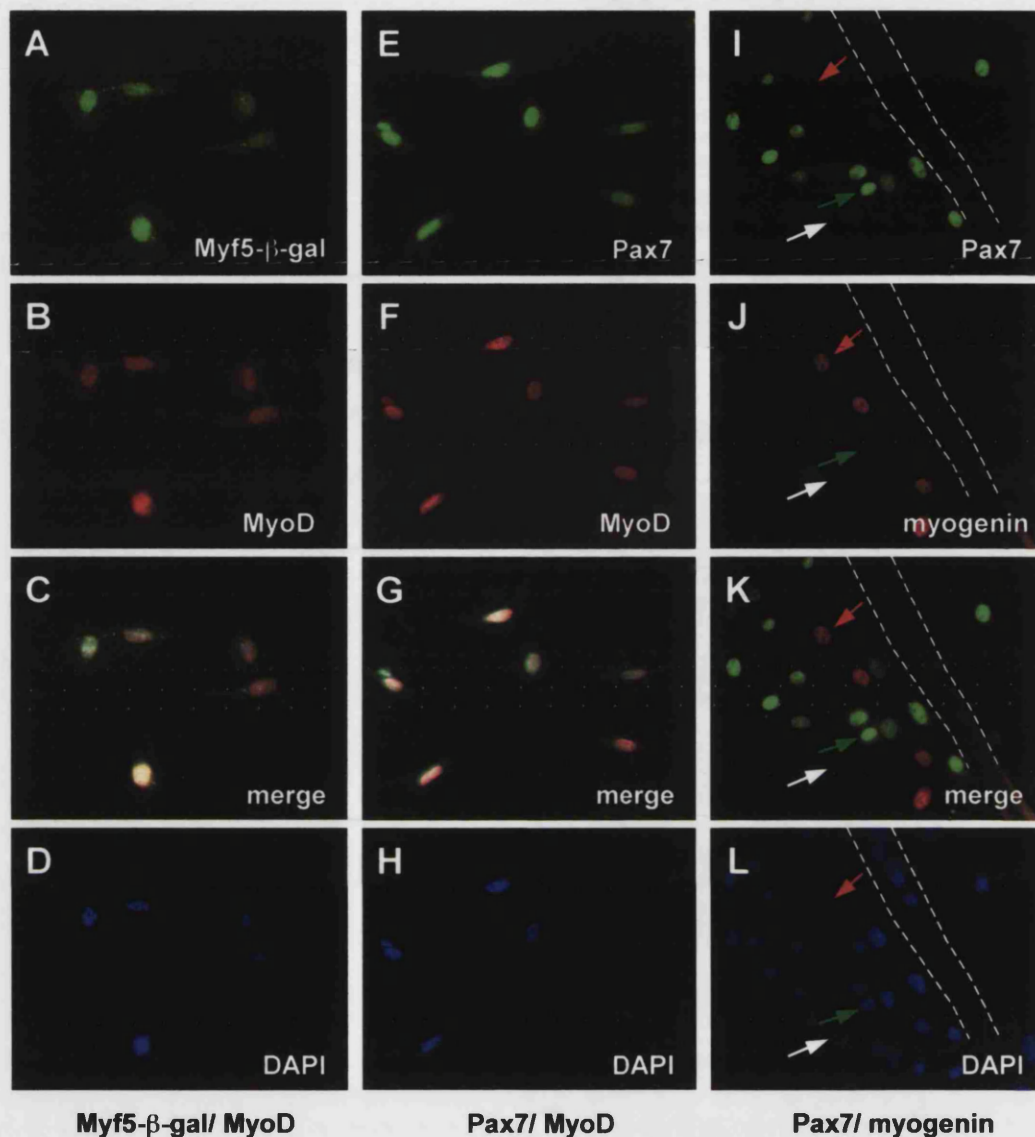


Fig. 4.2. Fluorescence immunocytochemistry of clone Myf5E2 satellite cell-derived myoblasts. Proliferating mononucleate cells stained for β -gal and MyoD (A-D) and Pax7 and MyoD (E-H). Almost all cells express Pax7, MyoD and, at varying levels, Myf5- β -gal. Differentiated culture stained for Pax7 and myogenin (I-L). Pax7 expression is lost by nuclei that have become incorporated into a myotube (outlined), but is retained by many non-fused mononucleate cells (green arrows). Myogenin is expressed by some cells (red arrows), but is not co-expressed with Pax7 (K). Some DAPI⁺ mononucleate cells express neither marker (white arrows). Original magnification x400.

4.23 The potential of immortalised satellite cell-derived *Myf5*^{nLacZ} myoblasts to give rise to satellite cells

Clone Myf5E2 cells (4.21) were used to investigate the potential of a cloned cell line to generate anatomically and phenotypically-defined satellite cells. Myf5E2 cells were expanded *in vitro*, and immediately before grafting, trypsinised, washed in growth medium, and a viable cell count performed (2.22). Pellets of 5×10^5 cells were prepared. 3 *mdx*-nude host mice were anaesthetised (2.52) and 5×10^5 cells grafted into the pre-irradiated (2.53) TA muscle of each hindleg (2.54). Engrafted muscles were removed after 3 weeks. The left engrafted muscle of each mouse was processed for immunohistochemistry. Two serial sections were collected at 100 μ m intervals throughout about 2/3 of the muscle body. In the first set, fluorescence immunohistochemistry was used to detect expression of dystrophin protein (2.76). 3/3 engrafted muscles were found to contain 495 (\pm 194) dystrophin⁺ myofibres (Fig. 4.3).

The right engrafted muscle of each host mouse was disaggregated to obtain single myofibres (2.21). In contrast to preparations obtained for grafting (3.42), in this experiment myofibres with minor imperfections (i.e. small bends or branching) were not discarded, since it was thought that donor-derived satellite cells were most likely to be associated with recently-regenerated myofibres, which might be more sensitive to damage induced by the isolation process. However, myofibres with gross imperfections, such as areas of hypercontraction or adherent debris, were discarded. Single myofibres were either stained in X-gal solution to localise β -gal activity (2.77), or stained immunocytochemically for β -gal and Pax7 proteins (2.75) as described in Table 4.1. Single myofibres isolated from the EDL muscles of a *Myf5*^{nLacZ/+} mouse were used as positive controls (Fig. 4.1).

	Left engrafted TA	Right engrafted TA
mouse 1	Cryosectioning and dystrophin immunohistochemistry	Single myofibre isolation, immunocytochemistry for β -gal and Pax7
mouse 2	Cryosectioning and dystrophin immunohistochemistry	Single myofibre isolation, immunocytochemistry for β -gal and Pax7
mouse 3	Cryosectioning and dystrophin immunohistochemistry	Single myofibre isolation, X-gal and DAPI staining

Table 4.1. Analysis of muscles engrafted with 5×10^5 clone Myf5E2 conditionally-immortalised myoblasts.

Staining of myofibres from mouse 3 in X-gal solution localised β -gal activity to numerous nuclei. β -gal⁻ nuclei were strongly stained by DAPI, and β -gal⁺ nuclei were very weakly stained as a consequence of the quenching effect of the blue product of X-gal staining. In some newly-regenerated myofibres, chains of β -gal⁺ nuclei indicated that at least one myonucleus was of donor origin. Since myonuclear β -gal translocates to adjacent myonuclei contained within a common cytoplasm (Ralston & Hall 1989, Yang *et al.* 1997) it was not possible to distinguish donor-derived β -gal⁺ nuclei from closely-associated host myonuclei which had thus acquired β -gal expression. Conversely, some β -gal⁺ nuclei were identifiable as those of satellite cells by the absence of such translocation to adjacent DAPI⁺ myonuclei implying lack of cytoplasmic continuity with the parent myofibre (Heslop *et al.* 2001). Satellite cells were identified on both large, mature myofibres and smaller, centrally-nucleated myofibres (**Fig. 4.4**). Where several β -gal⁺ nuclei were located in association with a single myofibre, it was not possible to determine whether they were within the cytoplasm of the myofibre or within satellite cells.

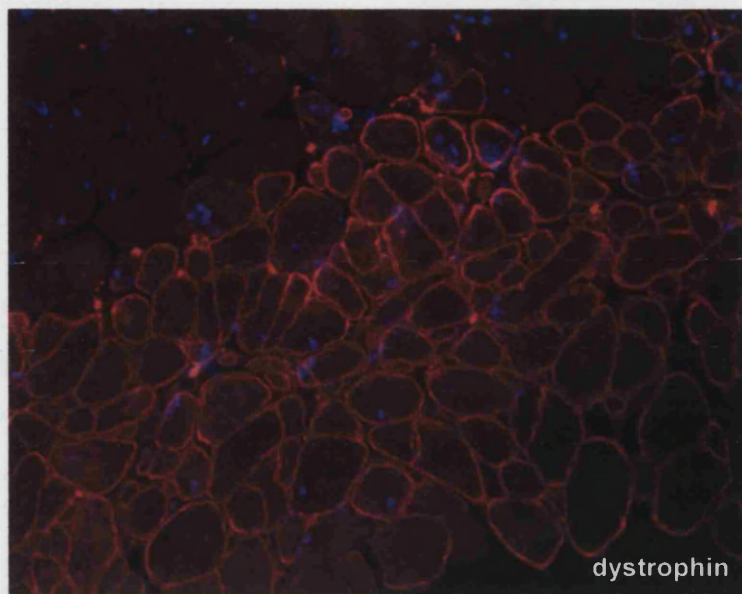


Fig. 4.3. Donor derived muscle formation in an *mdx*-nude TA muscle engrafted with 5×10^5 Myf5E2 satellite cell-derived myoblasts. Fluoresence immunocytochemistry for dystrophin protein, counterstained with DAPI. Original magnification x200.

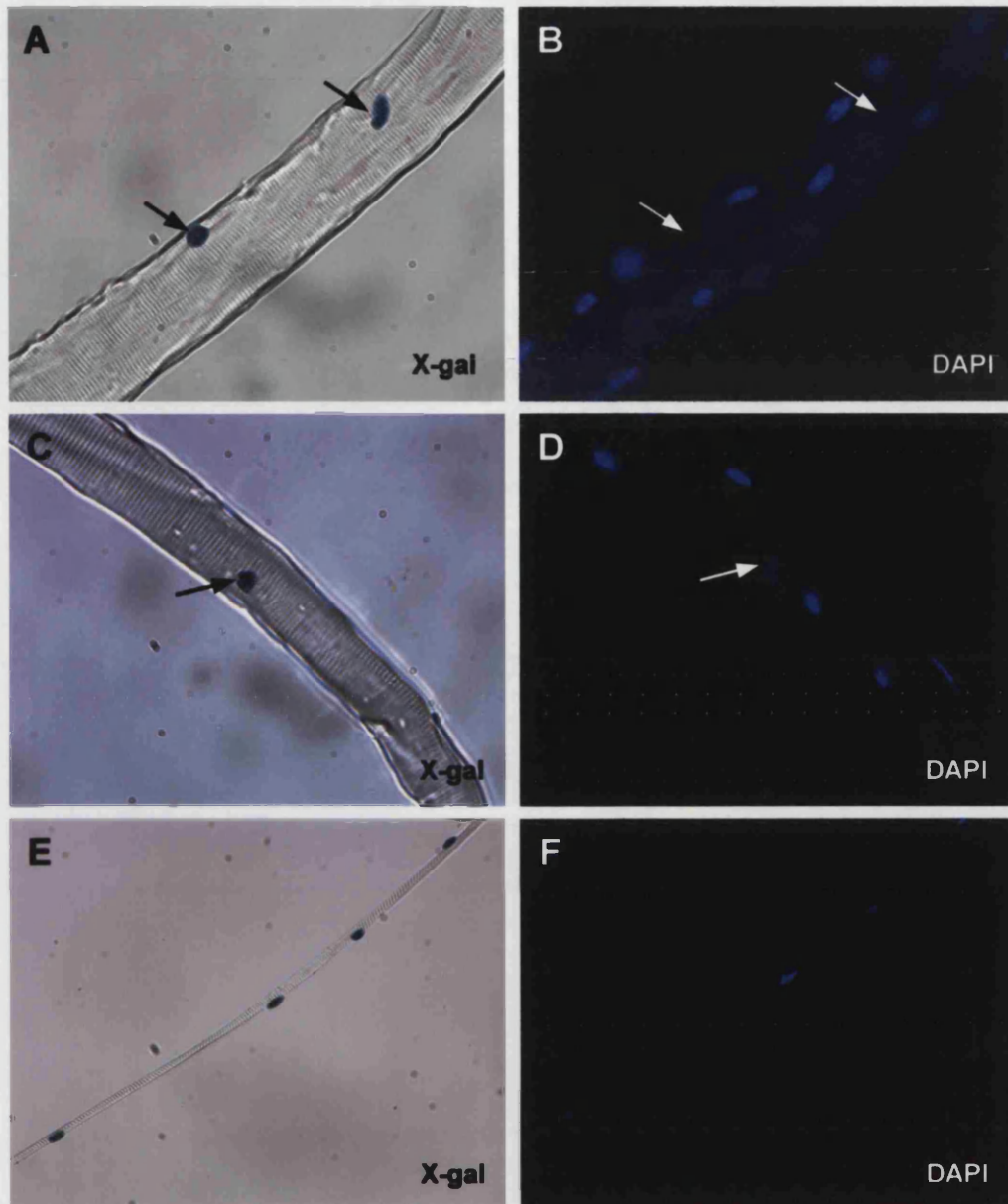


Fig. 4.4. Single myofibres isolated from an *mdx*-nude TA muscle engrafted with 5×10^5 Myf5E2 satellite cell-derived myoblasts. X-gal staining was used to localise expression of Myf5- β -gal fusion protein in donor-derived nuclei (A, C, E). Other nuclei were stained with DAPI (B, D, F). β -gal⁺ satellite cells (arrows) can be distinguished from β -gal⁺ myonuclei by the lack of translocation to adjacent myonuclei. Donor-derived satellite cells were seen on both mature myofibres (A-B) and relatively immature myofibres (C-D). Chains of β -gal⁺ nuclei in some immature myotubes indicated that one or more myonuclei were donor-derived (E- F). Original magnification x400 (A-D) or x200 (E-F).

Satellite cells were also identified in single myofibre preparations stained immunocytochemically for expression of Pax7 and β -gal proteins. Pax7 is expressed by the majority of satellite cell nuclei, but not myonuclei (Seale *et al.* 2000, Zammit *et al.* 2004a). Donor-derived satellite cells were identifiable by their co-expression of Pax7 and Myf5- β -gal fusion proteins (**Fig. 4.5**). Pax7⁺ β -gal⁻ satellite cells were presumed to be mainly host-derived. However, in the *Myf5^{nLacZ/+}* mouse, about 20% of anatomically-defined satellite cells do not express Myf5- β -gal fusion protein (Beauchamp *et al.* 2000), suggesting the possibility that in the present experiment, some Pax7⁺ β -gal⁻ satellite cells could have been donor-derived. Occasionally, chains of Pax7⁻ β -gal⁺ nuclei were seen in immature myofibres. Numbers of satellite cells were counted and are depicted in **Table 4.2** and **Fig. 4.6**.

Treatment with 18 Gy irradiation has been reported to reduce the total number of satellite cells that migrate from single myofibres after 40-60 h in culture to about 20% of normal values (LaBarge & Blau 2002), and to completely ablate their proliferative ability (Heslop *et al.* 2000, **3.72**). In a previous experiment, myofibres isolated from the non-irradiated (and non-dystrophic) TA muscles of 3F-*nLacZ*-2E TA mice were found to have a mean of 9.66 (± 0.20) associated Pax7⁺ satellite cells (**3.41**). Myofibres isolated from irradiated *mdx*-nude TA muscles in the present experiment had a mean of 1.81 (SE ± 0.67) Pax7⁺ β -gal⁻ satellite cells, 18.7% of the mean number associated with non-irradiated 3F-*nLacZ*-2E myofibres (**3.41**). These data are therefore in accordance with the finding of LaBarge & Blau (2002).

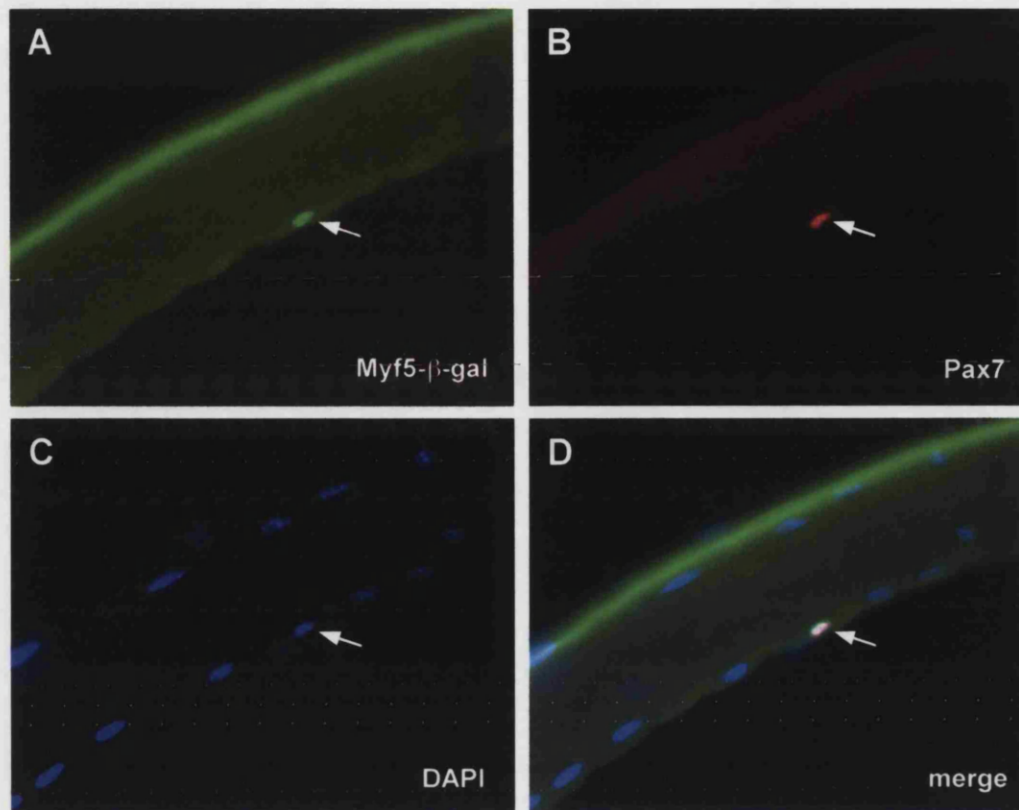


Fig. 4.5. Single myofibre isolated from an *mdx*-nude TA muscle engrafted with 5×10^5 Myf5E2 satellite cell-derived myoblasts. Immunocytochemistry for β -gal (A) and Pax7 (B) identifies a donor-derived satellite cell nucleus (arrowed) which co-expresses Myf5- β -gal fusion protein and Pax7 (D). Myonuclei are identifiable only by DAPI staining (C, D). Original magnification x400.

	mouse 1	mouse 2	total
Total myofibres	40	45	85
Total satellite cells	119	77	196
Pax7⁺ β-gal⁻ satellite cells	91	60	151
Mean Pax7⁺ β-gal⁻ satellite cells per myofibre	2.28 (±2.11)	1.33 (±1.62)	1.81 (SE ±0.67)
Pax7⁺ β-gal⁺ satellite cells	28	17	45
Mean Pax7⁺ β-gal⁺ satellite cells per myofibre	0.70 (±1.22)	0.38 (±0.86)	0.54 (SE ±0.23)
Percentage of myofibres with ≥1 β-gal⁺ satellite cells	35.0%	28.6%	31.8%
Percentage of β-gal⁺ satellite cells in whole population	23.5%	22.1%	22.8%
Estimated total satellite cells formed per graft^a	1.40 x 10 ³	0.76 x 10 ³	1.08 x 10³

^a Based on an assumption of 2 x 10³ total myofibres per engrafted muscle. The mean number of graft-derived satellite cells per sampled myofibre multiplied by the total myofibres in the engrafted muscle.

Table 4.2. Expression of Pax7 and β-gal proteins in satellite cells associated with myofibres isolated from two TA muscles engrafted with 5 x 10⁵ Myf5E2 cells.

Assuming that each engrafted muscle contained about 2 x 10³ total myofibres (typical for an irradiated *mdx* muscle at this timepoint, J. Morgan, personal communication), which had on average 0.54 donor-derived satellite cells per myofibre, Myf5E2 myoblasts generated an estimated 1.08 x 10³ new satellite cells per graft (**Table 4.2**). These data show that clonal populations of immortalised myoblasts can generate both differentiated muscle and anatomically-defined satellite cells which co-express the canonical satellite cell markers Pax7 and Myf5. However, since approximately 10³

satellite cells were formed from 5×10^5 Myf5E2 cells in each engrafted muscle, the frequency of satellite cell formation was only about 0.2% of the total grafted cells. Muscle formation from grafts of immortalised myoblasts is thought to originate from about 2% of grafted cells that survive and proliferate (Beauchamp *et al.* 1999). In the present study, muscle formation by the Myf5E2 clone was relatively efficient at 495 (± 194) dystrophin⁺ myofibres per graft, suggesting that the low efficiency of satellite cell formation was not due to inaccurate injections or poor myogenic potential of the cell line.

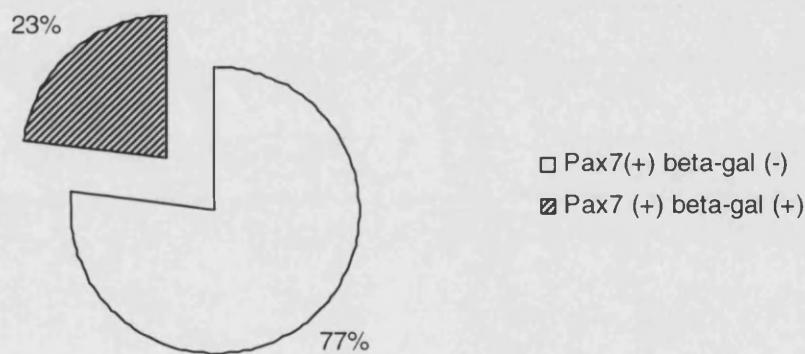


Fig. 4.6. Pie chart illustrating the contribution of Myf5E2 cell grafts to the satellite cell compartment of irradiated *mdx*-nude TA muscles (n=2). Almost all endogenous satellite cells express Pax7 (Zammit *et al.* 2004a). Single myofibres isolated from engrafted muscles were immunostained for Pax7 and β -gal proteins. Donor-derived satellite cells were identified by co-expression of Pax7 and Myf5- β -gal fusion protein.

The results of this experiment demonstrate that a clonal, conditionally-immortalised satellite cell-derived line is capable of both generating differentiated tissue and giving rise to satellite cells which express appropriate phenotypic markers.

4.3 The ability of satellite cells to generate new satellite cells

4.31 Aim

The objective of these experiments was to investigate the potential of satellite cells to renew their own compartment by generating new satellite cells.

4.32 Generation of reserve populations from satellite cells in culture

Previous studies have demonstrated that in tissue culture, both primary human myoblasts (Baroffio *et al.* 1996) and the C2C12 murine myoblast cell line (Yoshida *et al.* 1998) are capable of maintaining a proliferation-competent reserve population during differentiation (Baroffio *et al.* 1996, Yoshida *et al.* 1998). In addition, a study on isolated myofibres in suspension culture has recently shown that associated satellite cells generate clonal progeny with divergent fates. Within a single (probably clonal) cluster of cells, some progeny commit to differentiation as shown by expression of myogenin protein, whilst others fail to express myogenin and maintain expression of Pax7, thus constituting an undifferentiated reserve population (Zammit *et al.* 2004). Though when maintained as adherent cultures, almost all single myofibres generate differentiation-competent progeny (Rosenblatt *et al.* 1995), it is not known whether they similarly all generate populations of reserve cells. Using adherent myofibre cultures, the present experiment aimed to investigate the fates of satellite cell progeny several days after differentiation of the majority population.

Single myofibres were isolated from the EDL muscles of an 84-day-old C57 Bl/10 mouse and maintained as individual adherent cultures in the wells of chamber slides (2.21). Single myofibres were cultured for 192 h, with 50% medium changes at 48 h, 96 h and 144 h. Extensive proliferation occurred during the first 96 h after which satellite cell-derived myoblasts started to differentiate. By 192 h, the majority of mononucleate cells had fused into relatively large and mature myotubes. Cultures were fixed and fluorescence immunohistochemistry used to localise expression of myogenin and Pax7 proteins (2.74) (Fig. 4.7). The fusion index of each culture was calculated as previously described (2.43), except that nuclei were stained with DAPI instead of haematoxylin. Myogenin was expressed both in the nuclei of mononucleate cells and in some myonuclei. Pax7 expression was seen only in the nuclei of mononucleate cells. Counts were made in three random fields for each culture.

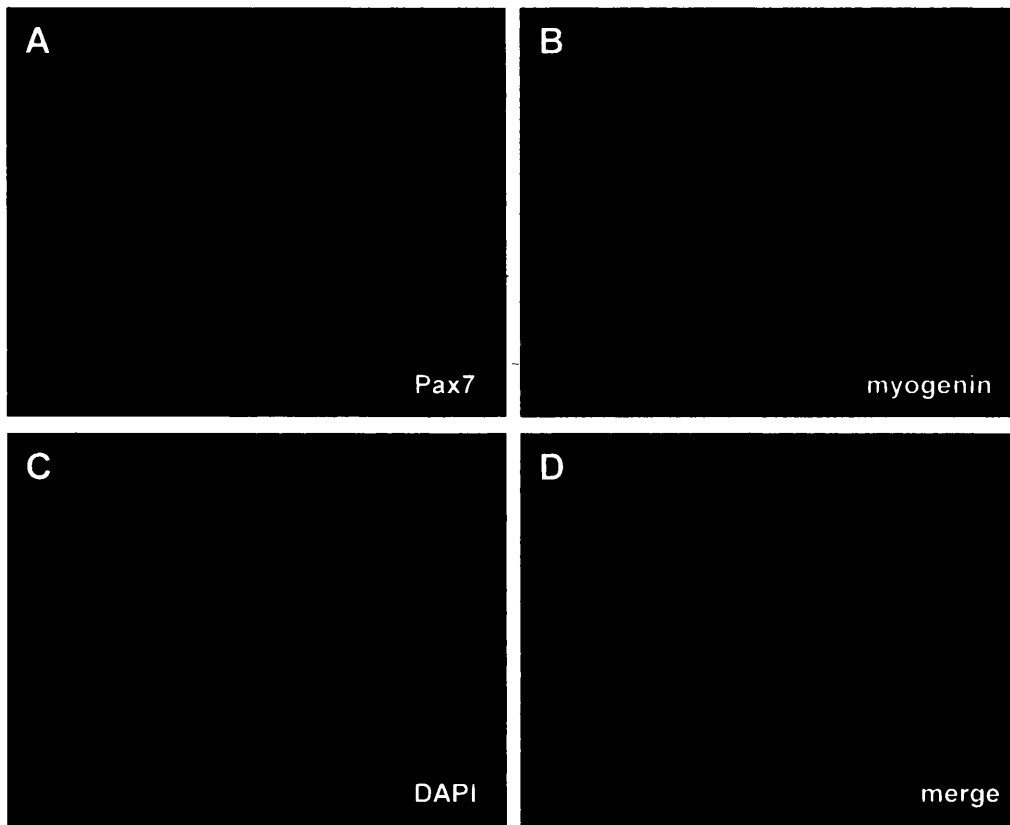


Fig. 4.7. Immunocytochemical detection of Pax7⁺ (green arrows) and myogenin⁺ (red arrows) mononucleate cells in a differentiated single myofibre-derived culture. After 8 days in culture, most satellite cell-derived myoblasts have fused into myotubes but some persist as a mononucleate undifferentiated reserve population. Co-expression of Pax7 and myogenin was not seen. Pax7⁻ myogenin⁻ cells may have been either myogenic cells that expressed neither marker at the time of sampling, or non-muscle contaminants. Original magnification x100.

No co-expression of myogenin and Pax7 was seen. Cultures (n=10) contained 76.5% (± 16.1) nuclei within myotubes, 12.5% (± 5.7) Pax7⁺ mononucleate cells and 4.7% (± 3.0) myogenin⁺ mononucleate cells. The 6.3% (± 14.0) of total nuclei within Pax7⁻ myogenin⁻ mononucleate cells were probably myogenic precursors which at the time of sampling did not express either of the markers examined, or less likely, non-muscle contaminants (**Figs. 4.7 & 4.8**). These data show that several days post-differentiation, all (10/10) cultured single myofibres generated cells that persisted in a mononucleate and undifferentiated state. Pax7⁺ mononucleate cells may be analogous with satellite cells *in vivo* (Seale *et al.* 2000, Zammit *et al.* 2004). Myogenin⁺ cells may represent the differentiation-committed precursor population identified by Rantanan *et al.* (1995), or may be terminally differentiated myocytes which failed to fuse in the tissue culture environment.

In a previous study, satellite cells maintained in association with single myofibres were found to give rise to progeny that adopted divergent fates with a mechanism suggestive of self-renewal (Zammit *et al.* 2004a). Here, a Pax7⁺ satellite cell-like phenotype was generated from populations of cells which had exited their sub-laminal position and thus were not maintained in association with myofibres. These data therefore suggest that undifferentiated reserve cells might initially be formed by a myofibre-independent mechanism, and only later recruited into the sub-laminal satellite cell niche.

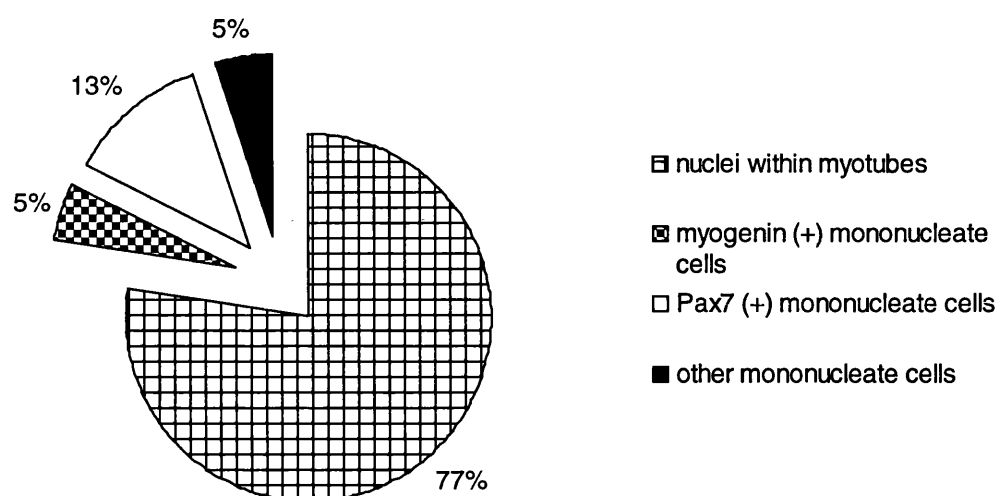


Fig. 4.8. Pie chart showing the divergent fates of satellite cell-derived MPC in EDL single myofibre cultures 8 d after explant (n=10). The majority of cells fused into myotubes, however some remained mononucleate and expressed either Pax7 or myogenin, but not both (**Fig. 4.7**). Some mononucleate cells were negative for both these markers. Pax7⁺ cells may be analogous with satellite cells *in vivo* (Seale *et al.* 2000, Zammit *et al.* 2004a). Myogenin⁺ cells may represent the differentiation-committed precursor population identified *in vivo* by Rantanen *et al.* (1995), or may be terminally differentiated cells which failed to fuse in the tissue culture environment. Unstained mononucleate cells are likely to be myogenic cells that at the time of sampling did not express either of the markers examined, but could alternatively be non-muscle contaminants.

4.33 The potential of grafts of *Myf5^{nLacZ/+}* single myofibres to give rise to satellite cells

(a) Identification of satellite cells derived from grafts of EDL, soleus and TA myofibres

Grafts of myofibres derived from the muscles of *Myf5^{nLacZ/+}* mice were used to investigate the ability of satellite cells to generate new anatomically- and phenotypically-defined satellite cells *in vivo*. Single myofibres were isolated from the EDL, soleus and TA muscles of donor 42 to 49-day-old *Myf5^{nLacZ/+}* mice and prepared for grafting as previously described (2.21, 3.42). Myofibres were grafted into the pre-irradiated TA muscles of *mdx*-nude mice (2.53, 2.55). After three weeks, engrafted muscles were removed and disaggregated into single myofibres (2.21), and X-gal staining used to localise β -gal activity (2.77). The undigested part of each engrafted TA muscle body was similarly fixed and stained in X-gal.

To maximise the chances of obtaining a positive result, in a preliminary experiment grafts of five, rather than single EDL myofibres were used. Of a total of 16 engrafted muscles, no isolated myofibres were stained by incubation in X-gal. However, 1 of 16 muscle bodies contained nuclear-localised β -gal expression. Under a dissecting microscope, β -gal-expressing myofibres were dissected out of this muscle using fine forceps and pins. The fragility and small size of these myofibres (estimated at ~1 mm) and made the process difficult; consequently, only four relatively-intact β -gal-expressing myofibres were obtained, a small sample of the total. Of these, two myofibres contained several β -gal⁺ nuclei which may have been either myonuclei, or within satellite cells. The other two myofibres contained one and two strongly β -gal⁺ nuclei respectively. These two myofibres were stained with DAPI to visualise all nuclei. The presence of several adjacent DAPI⁺ β -gal⁻ nuclei showed that the β -gal activity was localised to the nuclei of satellite cells and not to myonuclei. It has been well-established that myonuclear β -gal translocates to adjacent myonuclei contained within the cytoplasm of the same myofibre (Ralston & Hall 1989, Yang *et al.* 1997). Satellite cells were therefore identifiable by the absence of such translocation implying lack of cytoplasmic continuity (Heslop *et al.* 2001). However, where several closely associated β -gal⁺ nuclei were seen in a single myofibre, it was not

possible to determine whether they were within satellite cells, or alternatively were myonuclei.

In subsequent experiments grafts of single myofibres were used. A proportion of grafts contained β -gal⁺ nuclei in both myofibres isolated by collagenase digestion, and within the undigested part of the TA muscle body (**Table 4.3**). All successful grafts generated both unequivocally identifiable β -gal⁺ satellite cell nuclei, myonuclei, and many nuclei whose location was indeterminable. Donor-derived satellite cells were seen on both large, mature myofibres, and smaller, centrally-nucleated myofibres which were likely to have been recently regenerated. Chains of β -gal⁺ nuclei in small, immature myofibres indicated that one or more myonuclei were donor-derived (**Fig. 4.9**). Only a proportion of myofibres containing β -gal⁺ nuclei were obtainable intact, and only a small number of probable satellite cell nuclei could be unequivocally characterised as such. However, subjectively, the number of donor-derived satellite cells appeared to greatly exceed the number originally present in the graft (see **Fig. 4.10A**). β -gal⁺ nuclei appeared to be more numerous and distributed over a larger area when derived from EDL or soleus myofibre grafts than when derived from TA myofibre grafts. Since at this timepoint, successful EDL and soleus myofibre grafts generate larger numbers of donor-derived myofibres than TA myofibre grafts (**3.4**), this suggests that donor-derived satellite cells may have been formed with a frequency proportional to the amount of new muscle generated.

Graft derived from <i>Myf5</i> ^{nLacZ/+} muscle	No. of grafts	Grafts generating β -gal ⁺ satellite cells
5 x EDL myofibres	16	1 (6.3%)
EDL single myofibre	10	2 (20%)
soleus single myofibre	6	2 (33.3%)
TA single myofibre	10	5 (50%)

Table 4.3. Frequency of satellite cell formation by grafts of *Myf5*^{nLacZ/+} myofibres. β -gal activity was localised by incubation in X-gal.

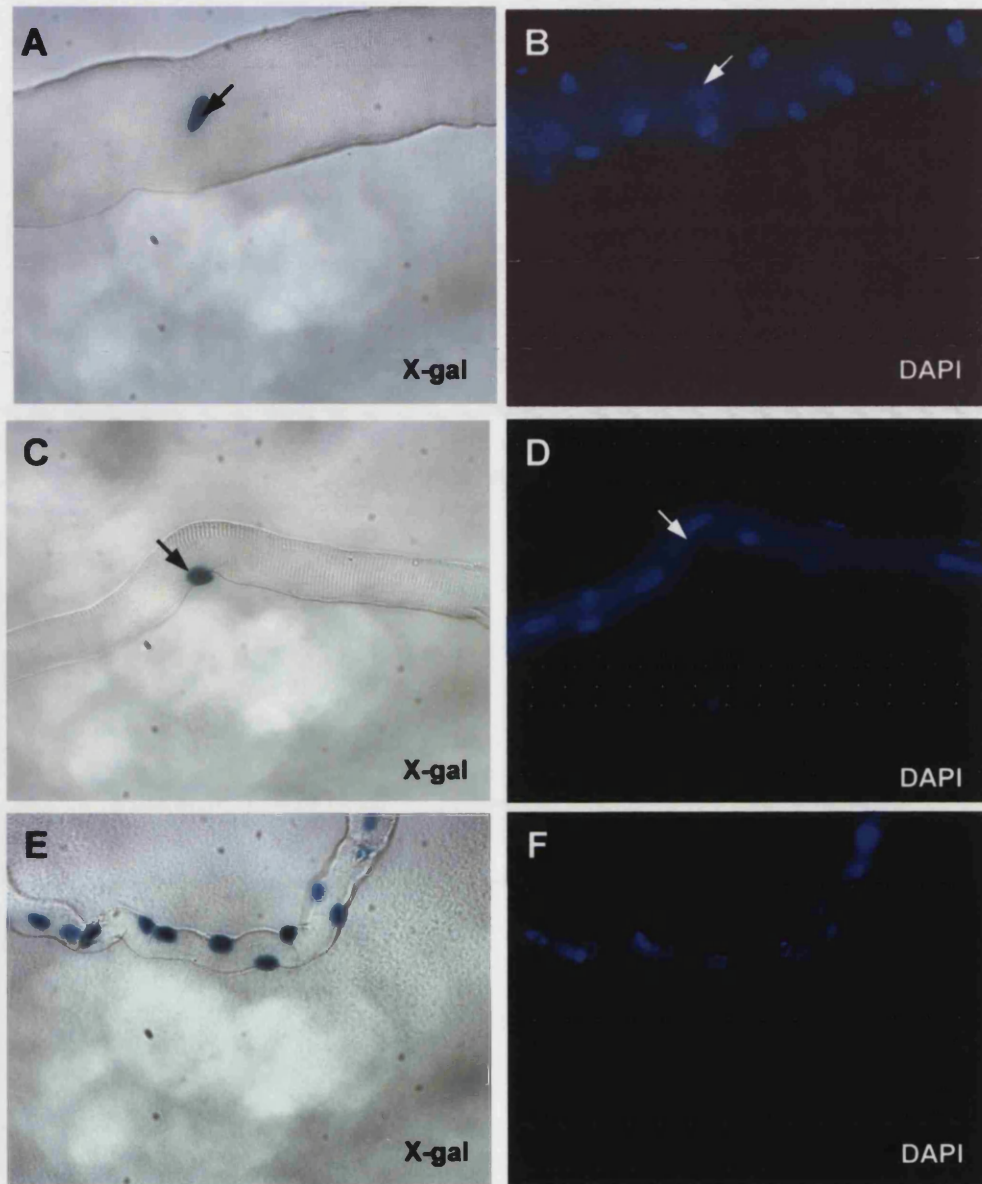


Fig. 4.9. Single myofibres isolated from *mdx*-nude TA muscles engrafted with a single *Myf5^{nLacZ/+}* EDL myofibre. X-gal staining localises Myf5-β-gal fusion protein to donor-derived nuclei (A,C, E). Other nuclei are stained with DAPI (B, D, F). β-gal⁺ satellite cells (arrows) are distinguished from β-gal⁺ myonuclei by the lack of translocation to adjacent myonuclei. Donor-derived satellite cells were seen on both mature myofibres (A-B) and immature myofibres (C-D). Chains of β-gal⁺ nuclei in some immature myotubes indicated that one or more myonuclei were donor-derived (E- F). A-D were originally isolated as viable single myofibres. E-F was dissected from the fixed and stained muscle body. Original magnification x400.

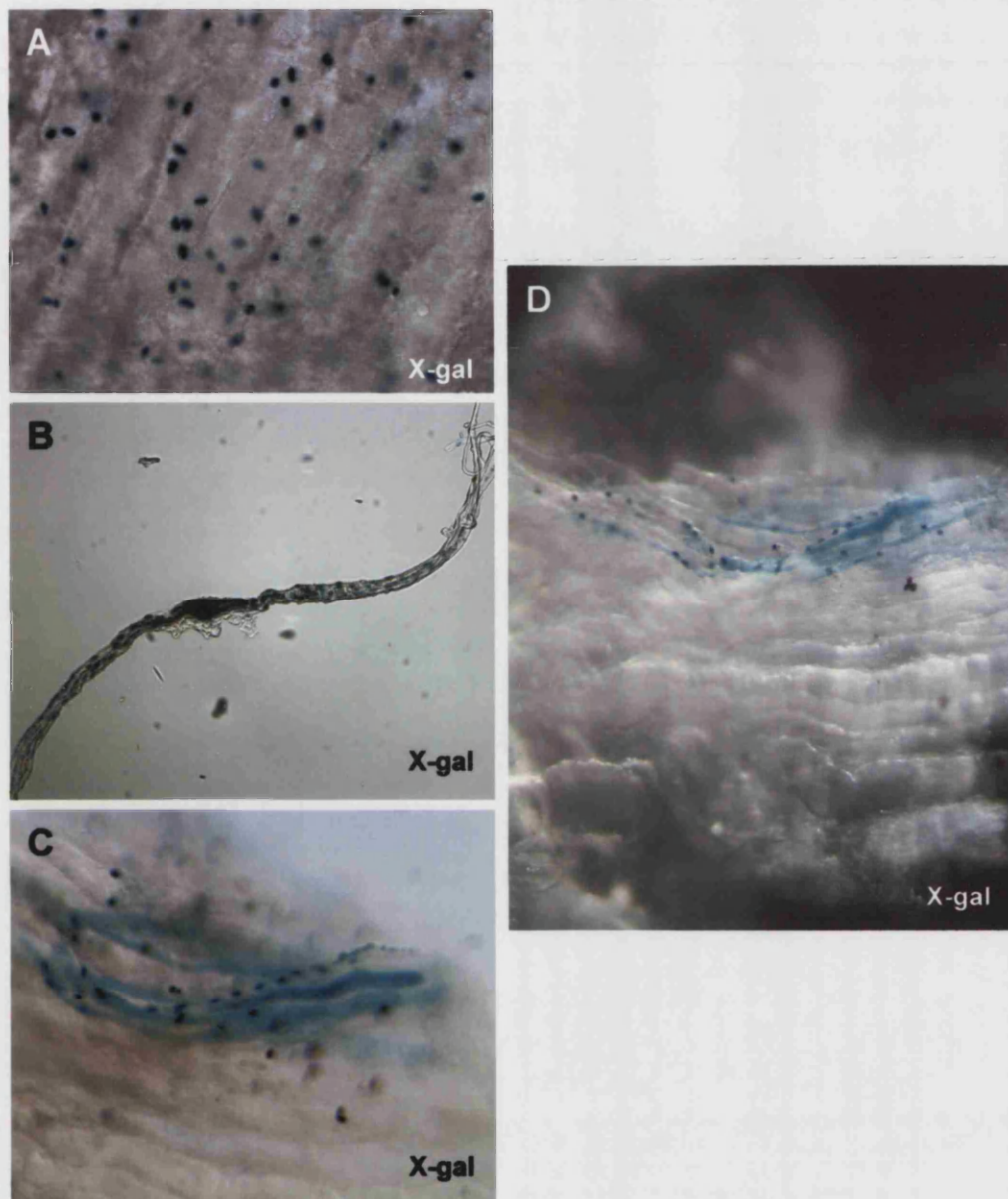


Fig. 4.10. Can grafted satellite cells contribute to muscle spindle regeneration? Most *Myf5^{nLacZ/+}* myofibre-engrafted muscles exhibited a pattern of X-gal staining similar to that shown in (A). In one muscle engrafted with a soleus single myofibre, however, a group of myofibres exhibited an unusual pattern of staining after incubation in X-gal (C, D). The staining was reminiscent of that seen in muscle spindles isolated from *Myf5^{nLacZ/+}* muscles (B), suggesting the possibility that satellite cells may have the potential to contribute to spindle regeneration. Original magnification x200 (A, C) or x100 (B, D).

Grafts of five myofibres generated satellite cells less frequently than grafts of one single myofibre (**Table 4.3**). Although five myofibres could be drawn into a glass needle relatively easily, when expelled they were likely to have become tangled and damaged, perhaps hypercontracting. The decreased viability of myofibres may have affected the viability or potential of their associated satellite cells.

In one soleus myofibre-engrafted muscle, after incubation in X-gal a pattern of β -gal expression similar to that of *Myf5^{enLacZ/+}* muscle spindles (Zammit *et al.* 2004) was seen. In the absence of staining for specific markers such as spindle-specific MyHC isoforms (Soukup & Thornell 1997), it could not be determined unequivocally whether this staining indicated a contribution to spindle regeneration (**Fig. 4.10**), however, this could be a possibility.

(b) Quantification of Pax7⁺ satellite cells derived from grafts of soleus single myofibres

Single myofibres were isolated from the soleus and TA muscles of a donor 43-day-old *Myf5^{enLacZ/+}* mouse (**2.21, 3.42**) and one myofibre grafted into each of the bilateral pre-irradiated TA muscles of three *mdx*-nude mice (**2.53, 2.55**). After three weeks, engrafted muscles were removed and disaggregated into single myofibres (**2.21**). Isolated myofibres were stained immunocytochemically for Pax7 and β -gal proteins (**2.74**) (**Fig. 4.11**). The undigested part of the engrafted TA muscle body was stained in X-gal solution (**2.77**).

After incubation in X-gal, 2/6 muscle bodies were found to have nuclear-localised β -gal activity (**Fig. 4.10A**). 4/6 single myofibre preparations were found to contain Pax7⁺ β -gal⁺ satellite cells, counts of which are shown in **Table 4.4** and **Fig. 4.12**. Overall, 5/6 (83%) of engrafted muscles contained donor-derived cells identifiable either by X-gal staining of the undigested body, immunocytochemistry of isolated myofibres, or both (**Table 4.4**).

Counts of satellite cells for individual engrafted muscles were likely to have been subject to either positive or negative sample bias. Where satellite cells were formed on single myofibres located in the centre of the muscle, they were unlikely to have been isolated in single myofibre preparations. Conversely, where satellite cells were

formed close to the periphery of the muscle, they were very likely to have been included in the sample. Of the six engrafted muscles, only one contained satellite cells identifiable by both X-gal staining of the muscle body and immunocytochemistry of single myofibres. One muscle contained cells only identified by X-gal staining of the muscle body, and three contained satellite cells only identified by immunocytochemistry of isolated myofibres (**Fig. 4.11**). Although counts derived from individual muscles could therefore potentially be either over- or underestimates, data pooled from the total counts derived from all six muscles are likely to be representative of the overall trend (**Table 4.4**).

An estimated 0.47×10^3 Pax7⁺ β -gal⁺ satellite cells were formed per graft of 22 (**3.42**) soleus satellite cells (**Table 4.4**) (**Fig. 4.12**). The estimated frequency of satellite cell formation was therefore about 2000% of the original number of grafted cells. In contrast, grafts of Myf5E2 cells had an estimated frequency of satellite cell formation which was about 0.2% of the total grafted cells (**4.23**). In Chapter 3, it was shown that satellite cells in single myofibre grafts generate new muscle more efficiently than grafts of immortalised myoblasts (**Table 3.6**). Similarly, the results of the present experiment demonstrate that satellite cells in single myofibre grafts generate new satellite cells with much greater efficiency than immortalised myoblasts.

In previous experiments, 75% of soleus myofibre grafts were found to give rise to differentiated muscle (**3.45**), and the results of the present experiment demonstrate that 83% of grafts give rise to satellite cells by the same timepoint. This indicates that most grafts generate both differentiated muscle and satellite cells. Pax7⁺ β -gal⁺ satellite cells were observed on both large, mature myofibres, and more frequently, on small, centrally-nucleated myofibres that were probably still being augmented at the time of sampling (**Fig. 4.11**). Together, the empirical data and morphological observations strongly suggest that the cohort of satellite cells associated with each grafted single myofibre give rise to both new differentiated myofibres and new satellite cells concomitantly during the same regenerative event.

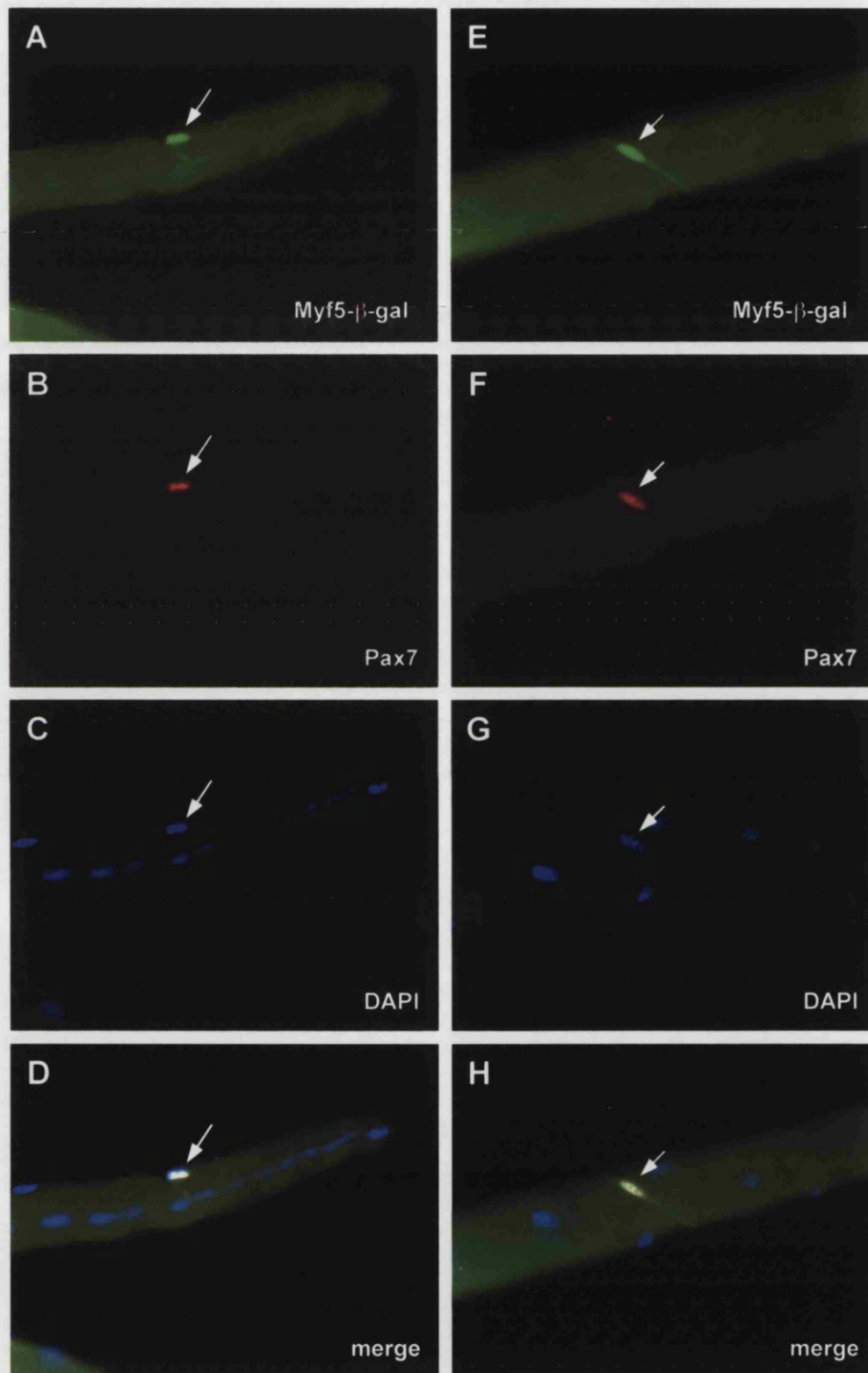


Fig. 4.11. Single myofibres isolated from *mdx*-nude TA muscles engrafted with a single *Myf5^{nLacZ/+}* soleus myofibre. Immunocytochemistry for β -gal (A, D) and Pax7 (B, F) identifies donor-derived satellite cells (arrowed) which co-express Myf5- β -gal fusion protein and Pax7 (D, H). Myonuclei are identifiable by DAPI staining (C, G). Original magnification x400.

	mouse 1 (RL)	mouse 1 (LL)	mouse 2 (RL)	mouse 2 (LL)	mouse 3 (RL)	mouse 3 (LL)	total
X-gal staining of undigested muscle body	no	yes	yes	no	no	no	2/6
Total isolated myofibres	27	40	31	34	25	29	186
Total satellite cells	31	55	ND	ND	50	70	ND
Pax7⁺ β-gal⁻ satellite cells	26	35	ND	ND	49	50	ND
Mean Pax7⁺ β-gal⁻ satellite cells per myofibre	0.96 (± 1.68)	0.88 (± 1.24)	ND	ND	1.96 (± 1.97)	1.72 (± 2.49)	ND
Pax7⁺ β-gal⁺ satellite cells	5	20	0	0	1	20	46
Mean Pax7⁺ β-gal⁺ satellite cells per myofibre	0.19 (± 0.40)	0.50 (± 1.30)	0	0	0.04 (± 0.20)	0.69 (± 1.23)	0.24 (SE ± 0.29)
Percentage of myofibres with ≥ 1 β-gal⁺ satellite cells	18.5%	22.5%	0	0	4.0%	34.5%	13.3%
Percentage of β-gal⁺ satellite cells in whole population	16.1%	36.4%	0	0	2.0%	28.6%	13.8%
Estimated total satellite cells formed per graft^a	0.38 x 10 ³	1.00 x 10 ³	0 ^b	0	0.08 x 10 ³	1.38 x 10 ³	0.47 x 10³

^a Based on an assumption of 2×10^3 total myofibres per engrafted muscle. The mean number of graft-derived satellite cells per sampled myofibre multiplied by the total myofibres in the engrafted muscle. ^b No Pax7⁺ β -gal⁺ satellite cells were identified in the isolated myofibre preparation, but the presence of β -gal activity in the undigested part of the muscle strongly suggests that some satellite cells had been formed.

Table 4.4. Expression of Pax7 and β -gal proteins in satellite cells associated with myofibres isolated from TA muscles engrafted with a single *Myf5^{nLacZ/+}* soleus myofibre. RL= right engrafted muscles. LL= contralateral left engrafted muscles. ND= not done.

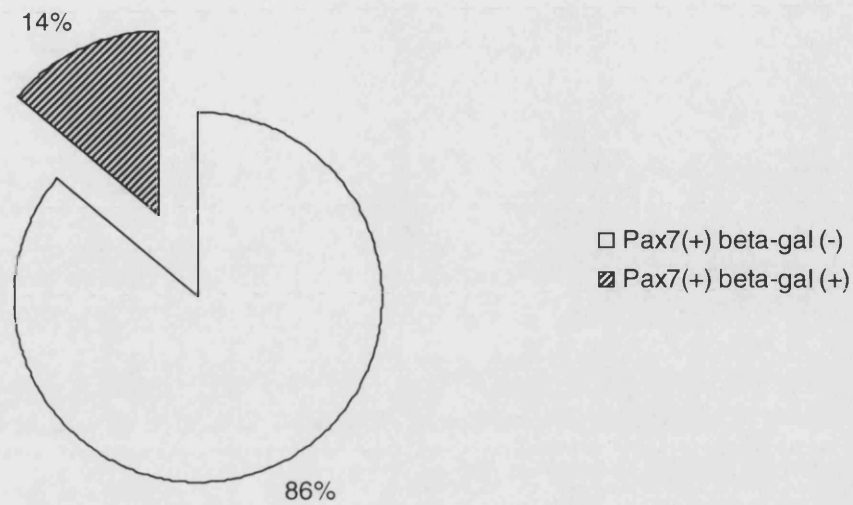


Fig. 4.12. Pie chart illustrating the contribution *Myf5*^{nLacZ/+} soleus single myofibre grafts to the satellite cell compartment of irradiated *mdx*-nude TA muscles (n=6). Single myofibres isolated from engrafted muscles were immunostained for Pax7 and β -gal proteins. Donor-derived satellite cells were identified by co-expression of Pax7 and Myf5- β -gal fusion protein.

The results of these experiments demonstrate that the proliferative progeny of satellite cells in single myofibre grafts adopt divergent fates, such that a subset of cells form a reserve population which returns to the satellite cell niche, and thereby renews the compartment. The spontaneous formation of satellite cell-like Pax7⁺ reserve cells in post-differentiated cultures further suggests that satellite cells are generated in a pre-programmed manner rather than solely in response to active recruitment by myofibres.

4.4 Formation of MPC by single myofibre grafts

4.41 Aim

In previous experiments, adult EDL myofibre grafts were found to form muscle (3.43) and satellite cells (4.32) infrequently. This low frequency was thought to result, at least partly, from a physiological rather than technical factor, since grafts of soleus myofibres formed muscle with much higher frequency (3.44). The apparent proportional relationship between the number of satellite cells present in the graft and the frequency of muscle formation (the proportion of grafts which generated β -gal⁺ nuclei) implies that donor-derived muscle may have been of clonal or oligoclonal origin (Table 3.5). However, the data did not exclude the possibility that donor-derived products were generated as a result of the synchronous activation and expansion of entire myofibre-associated satellite cell populations.

The clonality of muscle regeneration and satellite cell formation from grafts of conditionally-immortalised *H-2K^b*-tsA58 MPC has previously been studied by labelling cultured cells with unique retroviral inserts (Cousins *et al.* 2004). However, since retroviruses are only incorporated by actively mitotic cells, this approach was hampered by the fact that less than 100% of cells in primary cultures were labelled (personal communication, J. Morgan). Since the most significantly-myogenic MPC are those which do not incorporate ³H-thymidine prior to grafting (Beauchamp *et al.* 1999), this is a potentially significant limiting factor. In addition, the numerous unique insertion sites generated by infection of primary cultures were found to be difficult to resolve (Cousins *et al.* 2004). However, even with these caveats, the data generated by Cousins *et al.* (2004) demonstrated that muscle formation by grafted primary myoblasts was oligoclonal, rather than toticlonal. The original aim of the present experiment was to investigate the clonality of MPC formation by satellite cells in single myofibre grafts.

4.42 Experiment plan

The original plan was to infect the quiescent satellite cells in single myofibre preparations with a lentiviral vector containing a GFP gene (GFP lentivirus) (2.34), thereby labelling each satellite cell with a unique pattern of viral insertion sites. Labelled single myofibres would then be grafted into mouse muscles according to the

usual protocol. MPC derived from the graft would subsequently be isolated by enzymatic disaggregation and expanded in tissue culture. The clonality of these graft-derived cells would be analysed by identifying the number of unique viral insertion sites using linear amplification-mediated PCR (LAM-PCR). If cultures contained more than one insertion site, further analysis of the population would be carried out by generating several subclones. The single myofibre grafts would be derived from the *H-2K^b-tsA58* immortomouse (2.16) to facilitate expansion of graft-derived cells after subsequent *in vitro* conditional immortalisation. The experimental plan is shown in **Fig 4.13**.

Problems were encountered which prevented the whole of the experimental plan from being carried out. The LAM-PCR protocol was developed by Schmidt *et al.* (2002) for clonal analysis of retrovirally-labelled HSC engraftment in primates, where it was found to be a highly-sensitive technique which allowed identification of up to 80 clones within a population. Briefly, the technique involves linear amplification of target DNA with solid-phase second-strand synthesis, followed by ligation of an oligonucleotide cassette and then nested exponential PCR. Other workers within the laboratory were involved in a project which aimed to develop the LAM-PCR technique such that it could be used to analyse the clonality of cells derived from grafts of GFP lentivirus-infected myoblasts. Unfortunately, the project found that LAM-PCR produces inconsistent and unreliable results when applied to this type of experiment. Consequently, in the present experiment LAM-PCR could not be used to perform clonal analysis as was originally planned. However, without clonal analysis, the preliminary data were found to present some interesting features.

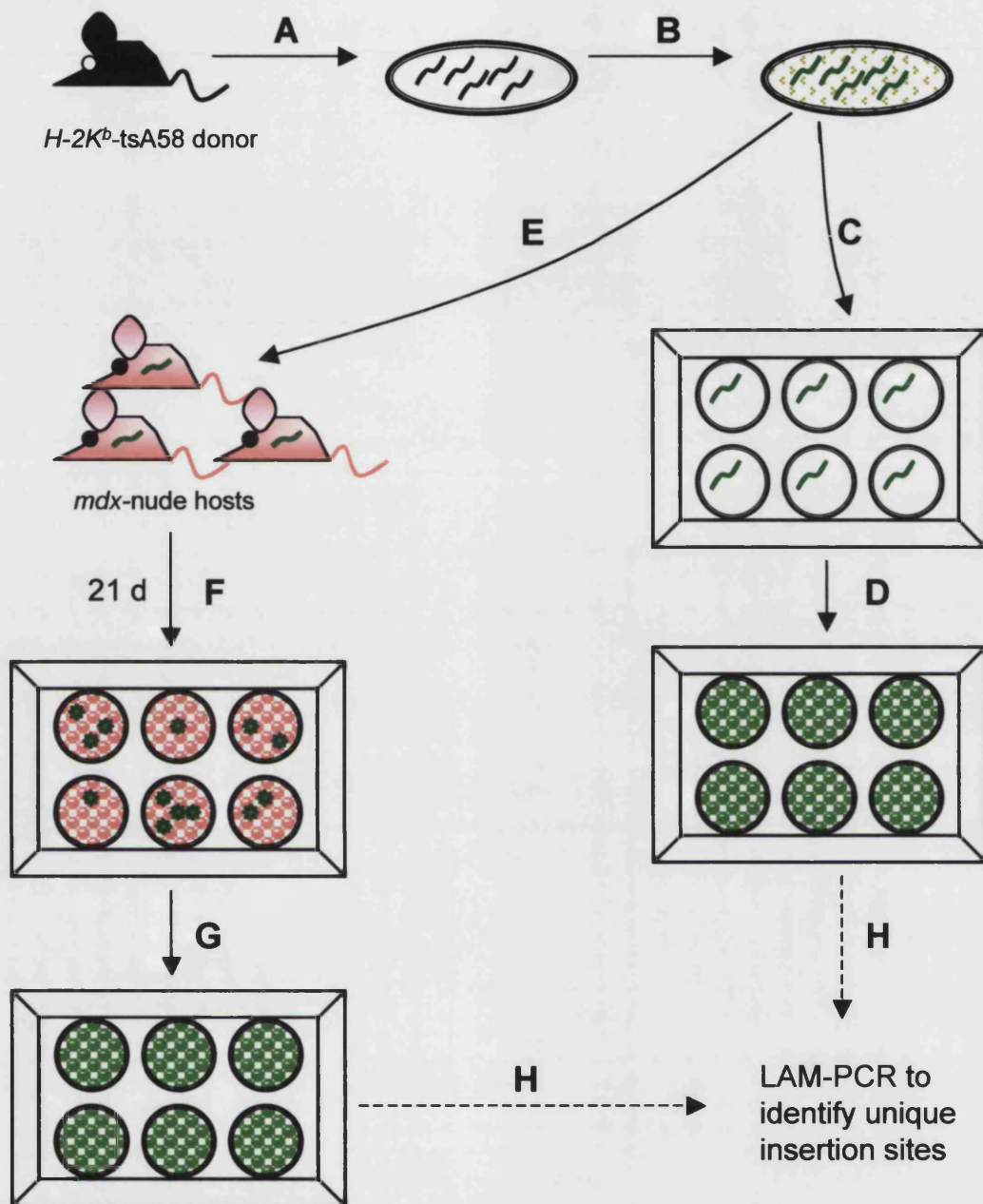


Fig. 4.13. Strategy for investigating the clonality of MPC formation by grafted satellite cells. Single myofibers isolated from EDL muscles of *H-2K^b-tsA58* donor mouse (A). Lentiviral infection of freshly-isolated myofibers (B). Culture of infected single myofibers (C) and expansion of satellite-derived cells under permissive conditions (D). Grafting of infected single myofibers into host TA muscles (E). Isolation of cells from engrafted muscles (F) and expansion and selection under permissive conditions (G). Use of LAM-PCR to identify number of unique insertion sites in *in vivo* and *in vitro* selected cells (H).

4.43 Grafts of GFP lentivirus-labelled *H-2K^b*-tsA58 single myofibres

Single myofibres were prepared from the EDL muscles of a male 112-day-old *H-2K^b*-tsA58 mouse (2.21) and labelled with GFP lentivirus (2.33, 2.34). A single labelled myofibre was grafted into each of the pre-irradiated TA muscles of seven *mdx*-nude mice (2.53, 2.55).

In addition, labelled myofibres from the same population were either fixed immediately after isolation, or maintained as suspension cultures in plating medium for 48 h, and then fixed. Immunocytochemistry of these myofibres for Pax7 and GFP showed that whilst at T:0 there was, as expected, no GFP expression, by T:48 both the myofibre and associated proliferating satellite cells contained cytoplasmic GFP protein (Fig. 4.14). This showed that the lentivirus had successfully integrated into at least a proportion of myonuclei and most satellite cell nuclei.

Some labelled myofibres were also maintained as adherent cultures (2.21). After three days, microscopic examination of cultures showed that satellite cells had emanated from myofibres and started to proliferate. Both the single myofibres and satellite cell progeny expressed cytoplasmic GFP. Single myofibres were removed from cultures. Cells were transferred to conditions permissive for tsA58 expression to induce conditional immortalisation (2.23). Six single myofibre-derived cultures were expanded, and aliquots preserved under liquid nitrogen. These populations were intended as controls to allow the numbers of clones generated by *in vitro* expansion to be compared with the numbers of clones generated by *in vivo* followed by *in vitro* expansion.

Single myofibre-engrafted muscles were removed after three weeks. Each muscle was individually disaggregated to obtain cell suspensions as previously described (2.22). To maximise the chances of obtaining graft-derived cells, the protocol was modified such that all three fractions were pooled and the suspension was not sieved. The cells derived from each muscle were cultured separately in small (75 cm³) flasks. By 72 h, some cells had adhered to the matrigel substrate, though no obvious proliferation had occurred. Cultures were then transferred to conditions permissible for tsA58 antigen expression, thereby inducing conditional immortalisation of graft-

derived cells (2.23). Cells were cultured for a further two weeks with medium changes at 72 h intervals. During this period, most cells appeared to die and detach from the substrate. This was as expected since most cells derived from the host would be rendered replication-incompetent after irradiation. However, after the first few days, clones of cells arose which had a healthy appearance and proliferated steadily. Aliquots of cells were seeded in chamber slides and cultured overnight before fixation and separate immunocytochemical stains desmin and T antigen (2.74). The numbers of cells found to express desmin and T antigen are shown in **Table 4.5**.

10/14 (71.4%) of cultures derived from engrafted muscles contained significant numbers (50-100%) of cells that expressed tsA58 antigen, a marker of donor origin (**Fig. 4.15**). Most cells in these cultures also expressed desmin (**Table 4.5, Fig. 4.15**).

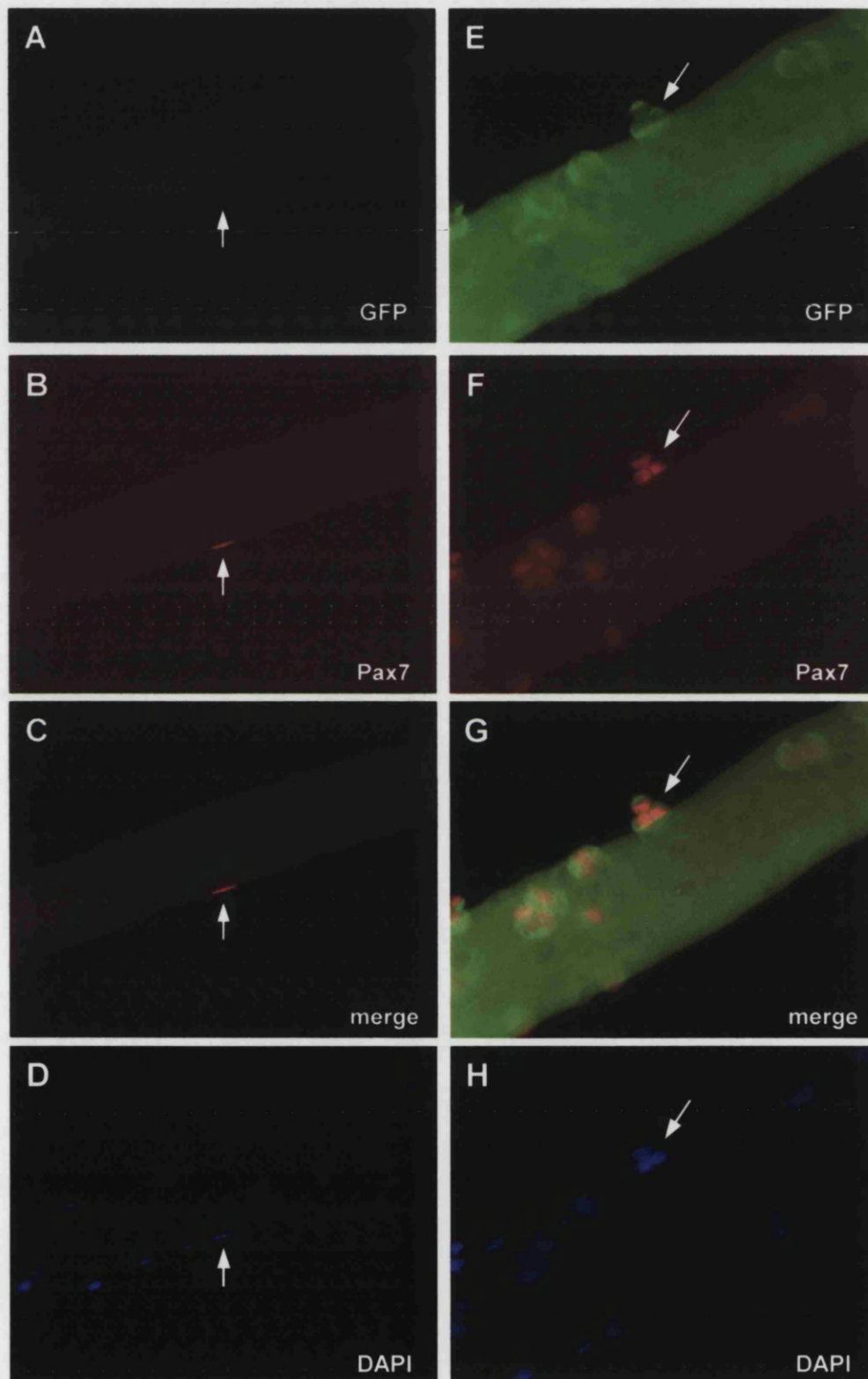


Fig. 4.14. Single myofibres infected with GFP lentivirus at T:0 (A-D) and T:48 (E-H) timepoints. Immunocytochemistry for Pax7 and GFP. At T:0, a the nucleus of a satellite cell is identifiable by Pax7 expression (arrows) but GFP is not expressed. By T:48, GFP is expressed in both the myofibre and clusters of proliferating Pax7⁺ satellite cells (arrows). Original magnification x400.

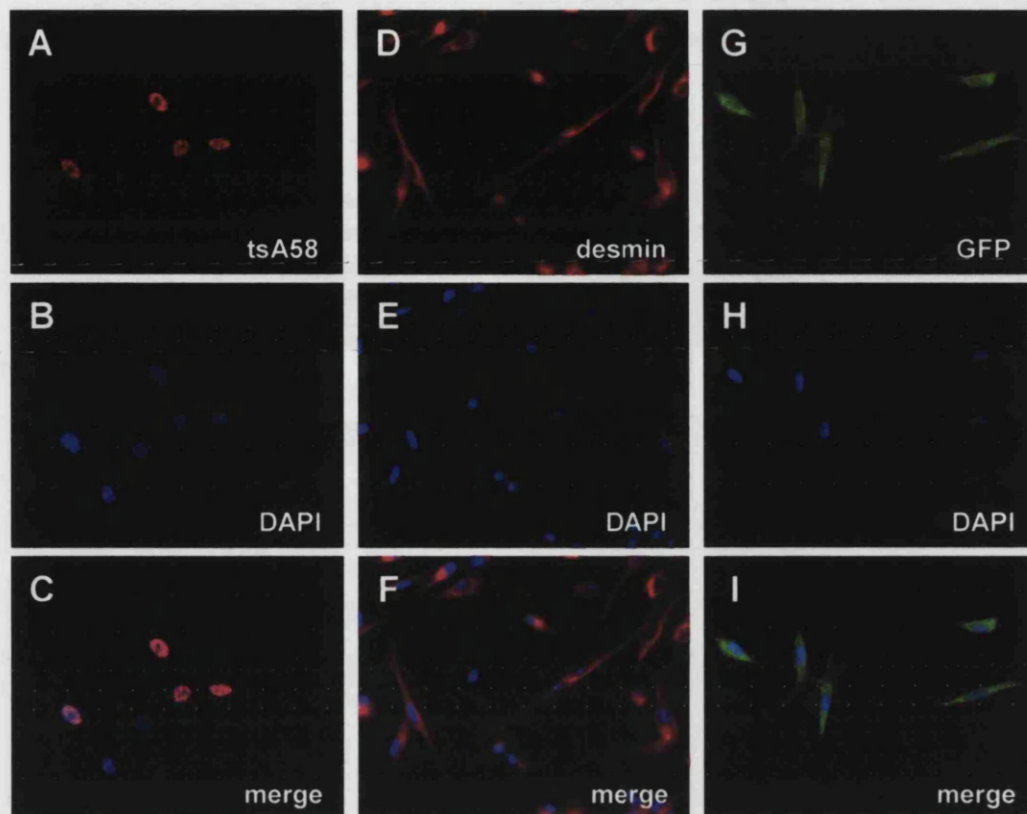


Fig. 4.15. Immunocytochemical detection of tsA58 antigen, a marker of donor origin (A-C), desmin, a marker of the myogenic lineage (D-F), and cytoplasmic GFP, a marker of successful lentiviral integration (G-I) in cells obtained by enzymatic disaggregation of muscles engrafted with a single *H-2K^b*-tsA58 EDL myofibre infected with GFP lentivirus. Original magnification x400 (A-C, G-I) or x200 (D-F).

Primary culture from engrafted muscle	tsA58 antigen ⁺ (% of cells)	Desmin ⁺ (% of cells)
1A	89.3	100
1B	ND	ND
2A	87.3	79.8
2B	100	96.3
3A	90.1	97.0
3B	2.1	50.0
4A	95.6	100
4B	87.8	90.9
5A	0	97
5B	0	92.1
6A	90.8	89.9
6B	50.0	66.7
7A	69.0	98.9
7B	85.2	92.6

Table 4.5. Expression of desmin and tsA58 antigen in primary cultures derived from muscles engrafted with *H-2K^b*-tsA58 EDL myofibres infected with GFP-lentivirus. Fluorescence immunocytochemistry for each antigen was carried out on separate aliquots of P1 cells. Cultures in grey italics were discarded as they contained few or no donor-derived (tsA58 antigen⁺) cells. ND= not done, due to absence of surviving cells.

Some cultures contained a relatively high frequency of desmin⁺ cells but no T antigen expression (**Table 4.5**). These cells were presumptively derived from host radiation-resistant cells (Heslop *et al.* 2000). Aliquots of cells from some cultures (at P2) were subsequently stained with an antibody against GFP. GFP protein was detected in most cells (**Fig 4.15**), showing that the foreign gene was expressed by cells that were proliferation-competent and had been expanded *in vitro*.

The presence of tsA58⁺ satellite cell-derived myoblasts in 10/14 (71.43%) cultures demonstrated that all of the corresponding single myofibre grafts had generated proliferation-competent progeny in engrafted muscles. This is surprising in light of the results of previous experiments in which only 20% of EDL myofibre grafts generated satellite cells (**4.33**) and 32% of grafts generated differentiated muscle (**3.43**). The extent to which proliferation occurred *in vivo* was not known, but it is

unlikely that the enzymatic disaggregation protocol would have been efficient enough to yield viable graft-derived cells if no proliferation had occurred.

The difference in frequency could be explained by the fact that in this experiment, single myofibres were incubated in lentiviral medium (containing 10% HS and 0.5% CEE) for 2 h prior to grafting. In a preliminary experiment, it was found that lentiviral integration did not occur in the absence of serum (data not shown). In all other experiments single myofibres were not exposed to serum and were grafted immediately after isolation. Exposure to serum may have activated previously quiescent satellite cells (Zammit *et al.* 2002), thereby causing them to enter an active myogenic program before insertion into the host muscle. Alternatively, some single myofibre grafts may generate proliferative progeny which do not differentiate into muscle and do not enter the satellite cell compartment. This suggests that in previously-described experiments (3.43, 4.43) some engrafted muscles either contained persistently quiescent graft-derived satellite cells, or alternatively, graft-derived proliferative phenotypes which were not identifiable by expression of Myf5- β -gal fusion protein.

The results of this experiment showed that the majority of EDL single myofibre grafts harbour satellite cells capable of generating significant numbers of MPC which are capable of extensive proliferation after subsequent re-isolation and conditional immortalisation *in vitro*. In addition, the finding that within single myofibre preparations, satellite cells can be isolated, infected at high frequency with a foreign gene, be grafted into a new muscle within a few hours of isolation, and subsequently both express the foreign gene and remain proliferation-competent, suggests that an efficient *ex vivo* gene therapy protocol could be designed around the single myofibre grafting technique.

4.5 The contribution of Pax3-GFP⁺ cells to satellite cells

4.51 Aim

This experiment formed part of a larger collaborative study carried out by Dr. D. Montarras and Dr. J.E. Morgan.

The paired box transcription factor Pax3 is a critical regulator of embryonic muscle development (Williams & Ordahl 1994, Tajbakhsh *et al.* 1997). In adult mice, *Pax3* expression is retained by a subset of satellite cells which occur frequently in the gracilis, diaphragm and certain forelimb muscles, but infrequently elsewhere (personal communication, D. Montarras). In the *Pax3*^{GFP/+} mouse (2.15), cytoplasmic GFP expression reports expression of the *Pax3* gene. Recent work carried out by Dr. D. Montarras and Dr. J.E. Morgan has shown that GFP⁺ cells sorted from *Pax3*^{GFP/+} diaphragms are highly myogenic *in vivo*. Grafts of 2 x 10⁴ freshly-isolated cells were found to generate 587 (±330) dystrophin⁺ myofibres in irradiated *mdx*-nude TA muscles (personal communication, J. Morgan). Pax3-GFP⁺ cells retain their phenotype after proliferation *in vitro*, suggesting that they may represent a distinct lineage of muscle precursors (personal communication, D. Montarras).

The objective of the present experiment was to investigate the ability of Pax3-GFP-sorted cells to both generate satellite cells and retain the Pax3-GFP⁺ phenotype.

4.52 Identification of satellite cells derived from grafts of Pax3⁺ cells

All cell isolation and sorting was carried out by Dr. D. Montarras on the day of grafting. 2 x 10⁴ Pax3-GFP⁺ diaphragm-derived cells were grafted into the right pre-irradiated TA muscles of two *mdx*-nude host mice (2.53, 2.54). Relatively small numbers of cells were used because of the difficulty in obtaining larger numbers. After three weeks, engrafted muscles were removed and enzymatically disaggregated to obtain single myofibres (2.21). Fluorescence immunohistochemistry using antibodies against GFP and Pax7 was carried out on isolated myofibres (2.75). Localisation of nuclear Pax7 protein marked satellite cells of both host and donor origin. Localisation of cytoplasmic GFP marked expression of nuclear Pax3 protein

in donor-derived satellite cells (**Fig. 4.16**). The numbers of satellite cells expressing Pax7 and/ or GFP were counted and are shown in **Table 4.6** and **Fig. 4.17**.

0.92 (SE ± 0.09) Pax3-GFP⁺ satellite cells were counted per myofibre. Assuming that the engrafted muscle contained 2×10^3 total myofibres, a mean of 1.79×10^3 satellite cells were therefore derived from each graft of 2×10^4 cells, a frequency of 9.0% (**Table 4.6**). The majority (94.4%) of the total 107 donor-derived satellite cells co-expressed Pax7 (**Table 4.6**, **Figs. 4.16 & 4.17**). The robust expression of GFP by the donor-derived satellite cells identified in this study (**Fig. 4.16**) shows that the Pax3⁺ phenotype can be retained in a host environment where endogenous Pax3⁺ cells are infrequent, and that it is not dependent on component of the niche from which the cells were isolated. Since it was not known whether GFP expression persisted in all donor-derived satellite cells, or just a subset, GFP⁻ Pax7⁺ satellite cells may have been of either host or donor origin. The number of Pax7⁺ satellite cells which did not express a marker of donor origin was, at 3.84 (SE ± 2.07) per myofibre, at least double that seen in previous comparable experiments (**4.22, 4.33**), and was greater than would be expected if 18 Gy irradiation reduced numbers to 20% of their normal value, as has been previously reported (LaBarge & Blau 2002).

This finding suggests the possibility that in the present experiment, the number of Pax7⁺ satellite cells was additionally augmented by donor-derived cells which did not express Pax3-GFP. However, it has been demonstrated that GFP-Pax3 cells retain their phenotype throughout prolonged tissue culture and until commitment to differentiation, strongly implying that Pax3⁺ Pax7⁺ MPC are lineage-distinct from Pax3⁻ Pax7⁺ MPC (personal communication, D. Montarras). In addition, the large count of Pax3⁻ Pax7⁺ satellite cells is mainly attributable to data derived from only one of the host animals, mouse 2 (**Table 4.6**). Therefore, although data generated by the present experiment do not exclude the possibility that Pax3⁺ Pax7⁺ cells are the earlier precursors of Pax3⁻ Pax7⁺ satellite cells, inter-animal variability is a more likely explanation for the discrepancy.

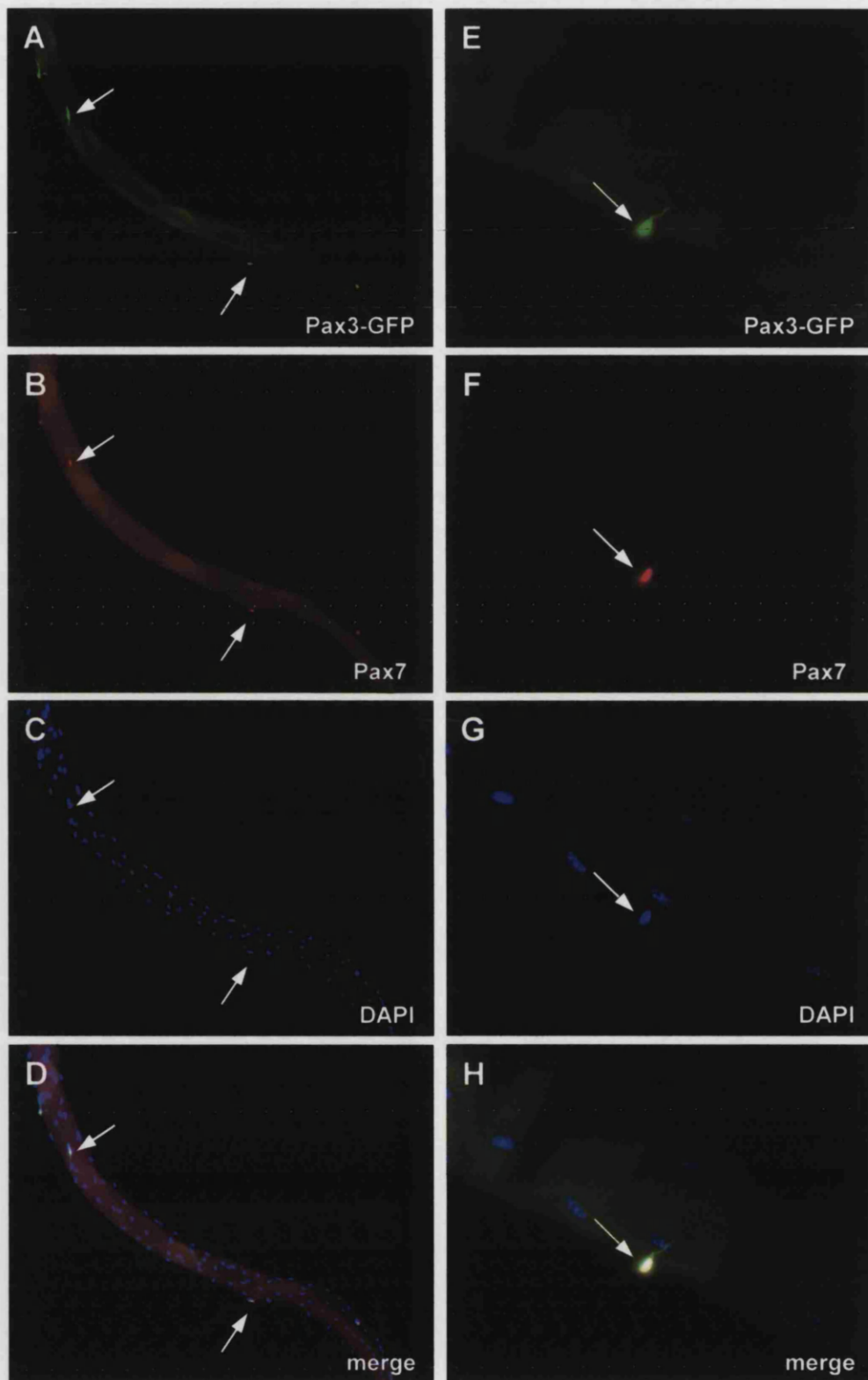


Fig. 4.16. Single myofibres isolated from an *mdx*-nude TA muscle engrafted with 2×10^4 Pax3-GFP⁺ cells sorted from the diaphragms of Pax3^{GFP/+} mice. Donor-derived satellite cells (arrowed) identified by expression of cytoplasmic GFP (A, D) and nuclear Pax7 (B, F). Counterstained with DAPI (C, G). Original magnification x100 (A-D) or x400 (E-H).

	mouse 1	mouse 2	total
Total myofibres	57	63	120
Total satellite cells	188	381	569
Pax7⁺ GFP⁻ satellite cells	133	329	462
Mean Pax7⁺ GFP⁻ satellite cells per myofibre	2.38 (±3.19)	5.31 (±5.21)	3.84 (SE ±2.07)
Pax7⁺ GFP⁺ satellite cells	53	48	101
Pax7⁻ GFP⁺ satellite cells	2	4	6
Total GFP⁺ satellite cells	55	52	107
Mean GFP⁺ satellite cells per myofibre	0.98 (±3.70)	0.86 (±2.30)	0.92 (SE ±0.09)
Percentage of myofibres with ≥1 GFP⁺ satellite cells	14.0%	27.0%	20.5%
Percentage of GFP⁺ satellite cells in whole population	29.3%	13.7%	18.8%
Estimated total satellite cells formed per graft^a	1.93 x 10 ³	1.65 x 10 ³	1.79 x 10³

^a Based on an assumption of 2 x 10³ total myofibres per engrafted muscle. The mean number of graft-derived satellite cells per sampled myofibre multiplied by the total myofibres in the engrafted muscle.

Table 4.6. Expression of cytoplasmic GFP and nuclear Pax7 proteins in satellite cells associated with myofibres isolated from two TA muscles engrafted with GFP-Pax3⁺ cells.

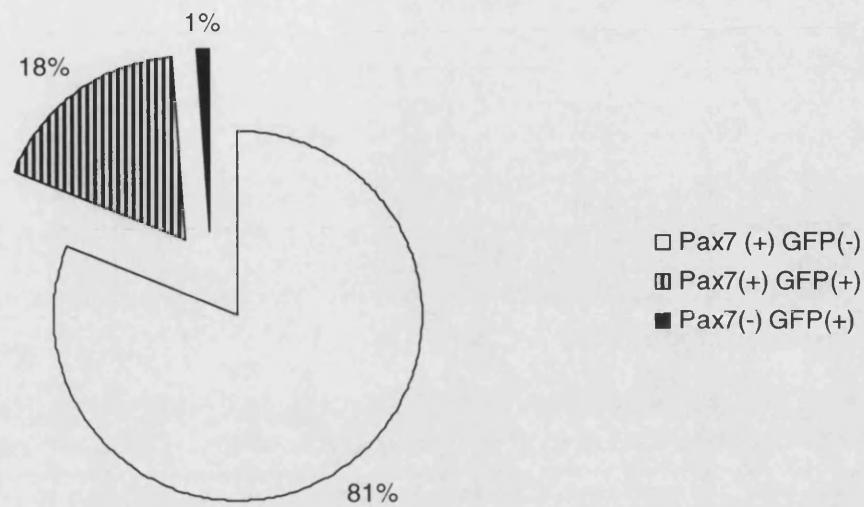


Fig. 4.17. Pie chart illustrating the contribution Pax3-GFP⁺ cell grafts to the satellite cell compartment of irradiated *mdx*-nude TA muscles (n=2). Almost all endogenous satellite cells express Pax7 (Zammit *et al.* 2004a). Single myofibres isolated from muscles engrafted with GFP-sorted cells were immunostained for Pax7 and GFP proteins. Pax7⁺ GFP⁻ cells could have been entirely host-derived, or may have included donor-derived cells which had ceased to express Pax3-GFP protein. GFP⁺ satellite cells are unequivocally donor-derived; most co-expressed Pax7 protein but a small subset did not.

The results of this experiment demonstrate that firstly, Pax3⁺ cells are capable of generating satellite cells, and secondly, that the Pax3-GFP⁺ phenotype is retained by cells that have acquired the satellite cell position. This suggests that Pax3⁺ myogenic cells form a separate lineage that is capable of maintaining its own population in a stem cell-like manner.

4.6 Comments on results

The experiments described in the chapter demonstrate that a conditionally-immortalised clonal cell line (4.2), primary isolated myoblasts (4.5) and myofibre-associated satellite cells (4.3) can generate new anatomically- and phenotypically-defined satellite cells after grafting. The data suggest that the ability to enter the satellite cell compartment and maintain or acquire expression of appropriate marker proteins is a quality common to myogenic phenotypes.

A previous study showed that the percentage of cells which survive in both conditionally-immortalised and primary myoblast grafts is about 2% (Beauchamp *et al.* 1999). Whilst Myf5E2 cells formed muscle reasonably efficiently (for an *H-2K^b-tsA58* cell line), at 495 (± 194) dystrophin⁺ myofibres per graft, the absolute number of donor-derived satellite cells generated in contralateral engrafted muscles was equivalent to no more than 0.2% of the total grafted population (4.22). These data therefore imply that the number of subclones which generate satellite cells is smaller than the number of subclones that generate differentiated muscle. The very large numbers of cells in the original graft made it impossible to determine whether the subclones which generated satellite cells had previously undergone proliferation and also generated muscle. A study which employed retroviral labelling as a means of studying the clonality of regeneration derived from myoblast grafts failed to find a correlation between clones that were significantly myogenic and those which generated satellite cells (Cousins *et al.* 2004). This presents the possibility that in grafts of Myf5E2 cells, satellite cells may have been formed from subclones which were stochastically recruited into a sub-laminal position, irrespective of their *in vivo* proliferative or myogenic potential. Hence though these data demonstrate that a satellite cell can generate a cell line capable of giving rise to anatomically- and phenotypically-defined satellite cells (Fig. 4.5), the formation of satellite cells under the experimental conditions described was not considered to be an unequivocal demonstration of self-renewal.

It has been shown previously that *in vitro*, Pax3-GFP expression is maintained by proliferating cells until commitment to differentiation, a finding which implies that Pax3⁺ cells are a distinct lineage rather than a transitory phenotype, or the more

primitive precursor of Pax3⁻ Pax7⁺ satellite cells (personal communication, D. Montarras). The data described in this thesis further show that proliferating Pax3⁺ cells can withdraw from the cycle without committing to differentiation and without adopting the more frequent Pax3⁻ Pax7⁺ satellite cell phenotype.

Grafts of 2×10^4 Pax3-GFP⁺ cells form comparable amounts of muscle to grafts of 5×10^5 Myf5E2 cells, and therefore do so with much greater efficiency. Whereas the absolute number of satellite cells generated from grafts of Myf5E2 cells was equivalent to only about 0.2% of the original population (4.22), grafts of Pax3-GFP⁺ cells were found to generate absolute numbers of satellite cells equivalent to 9.0% of the original population (4.52). Since only about 2% of cells are thought to survive grafting (Beauchamp *et al.* 1999), these data imply that at least a proportion of the clones which generated satellite cells had previously undergone proliferation, and probably participated in myogenesis. It is conceivable that the unusually high myogenic potential of Pax3-GFP cells⁺ (personal communication, J. Morgan) could be traced to improved survival in comparison to the cells examined by other studies (Beauchamp *et al.* 1999, Cousins *et al.* 2004). However, even if 100% survival is assumed, the number of nuclei required to generate several hundred myofibres and numerous satellite cells greatly exceeds the number of nuclei originally present in the graft, making proliferation a necessity. The results of this experiment therefore strongly imply that satellite cells were generated as part of a mechanism whereby a reserve population was actively maintained.

The derivation of Pax3-GFP⁺ cells from minced tissue prevented their origin from being defined unequivocally. Using a different gene-targeted mouse with a *LacZ* reporter, Pax3⁺ cells have been identified in the satellite cell position (Buckingham *et al.* 2003). However, it is not known whether the satellite cell compartment harbours the entire adult component of Pax3⁺ cells, or whether others exist outside the basal lamina. Therefore these data cannot be considered as a demonstration of satellite cell self-renewal. Notwithstanding, they strongly suggest that Pax3⁺ muscle-derived cells are a separate lineage which is capable of maintaining its own population during regenerative events.

Grafts of isolated single myofibres were used to examine the potential of defined satellite cells to generate new defined satellite cells (4.3). A proportion of grafts derived from EDL, soleus and TA muscles generated satellite cells identifiable by their discrete nuclear expression of Myf5- β -gal fusion protein. In addition, satellite cells derived from soleus myofibre were identified by immunocytochemistry for Pax7 and β -gal proteins. The progeny of grafts were also found to also contribute to newly-regenerated Myf5- β -gal⁺ myonuclei (Fig. 4.9). Satellite cells therefore became activated, proliferated to generate a larger pool of cells, and subsequently adopted divergent fates, either committing to differentiation or remaining mononucleate and becoming recruited into the satellite cell compartment. This finding formally demonstrates firstly, that the adult satellite cell compartment actively maintains itself by self-renewal, and secondly, that the satellite cells associated with one myofibre can generate progeny which contribute to the satellite cell populations of other myofibres, sometimes large numbers of other myofibres.

In vitro, all EDL single myofibres gave rise to progeny that were capable of both generating differentiated myotubes and maintaining a reserve population of undifferentiated satellite cell-like progeny (4.31). In principle, this implies that all EDL single myofibres should have the ability to generate satellite cells after grafting; in practice, only 20% (2/10) were found to do so. On exposure to serum-rich medium, the vast majority of satellite cells rapidly become activated (Zammit *et al.* 2002, 2003a) and in adherent myofibre cultures, many (or perhaps all) satellite cells subsequently proliferate (Rosenblatt *et al.* 1995, Zammit *et al.* 2002). This factor may explain the observed difference between the proportion of single myofibre grafts which generated MPC after exposure to serum (71.4%) (4.4) and the proportion of single myofibre grafts which generated satellite cells without exposure to serum (20%) (4.31).

In differentiated cultures, Pax7⁺ mononucleate cells formed 12.5% of the total nuclei (Fig. 4.8). Satellite cells form 2.79% of the total myofibre nuclei in single myofibre preparations (Zammit *et al.* 2002), less than a fifth of this value. The large counts of Pax7⁺ cells could either have been a tissue culture artefact, or could suggest that *in vivo*, not all reserve cells are destined to enter the satellite cell compartment. In a previously described experiment, the numbers of desmin⁺ cells obtained by enzymatic

disaggregation of EDL and soleus muscles was greater than the maximum number of satellite cells that could have been present in the preparation (**Table 3.1**). Speculatively, satellite cells may generate both new satellite cells, and other MPC which are outside the compartment at any given timepoint. The ability of satellite cell-like Pax7⁺ reserve cells to form outside the satellite cell niche in differentiating cultures suggests that reserve MPC may be actively generated prior to satellite cell formation, thus constituting the earlier precursors of anatomically-defined satellite cells. Alternatively, trafficking between satellite cells and non-satellite cell MPC might occur, whereby the two populations exist in a state of dynamic equilibrium.

In summary, the results of the experiments described in this chapter demonstrate that the adult satellite cell compartment maintains itself by a physiological process of self-renewal, whereby proliferative satellite cell progeny actively generate a reserve population of which some cells subsequently return to the satellite cell niche.

Chapter 5

The response of satellite cells to damage

5.1 Background and aim of study

5.11 Background to work

The function of stem cells is dependent on their characteristic ability to respond to the increased demands induced by damage, by upregulating proliferative activity and giving rise to differentiation-committed progeny that subsequently restore tissue integrity. When HSC are introduced into the circulatory system of a lethally-irradiated mouse, they respond by undergoing rapid clonal expansion to generate sufficient progeny to reconstitute the lymphohaematopoietic system and thus maintain viability (Till & McCulloch 1961, Smith *et al.* 1991, Osawa *et al.* 1996). After skeletal muscle tissue is severely injured by mincing (Studitsky 1964, Carlson 1970) or injection of myotoxins (Sadeh *et al.* 1985, Schultz & Jaryszak 1985, Heslop *et al.* 2000, Luz *et al.* 2002, reviewed Harris 2003), it rapidly regenerates. Histological analysis of myotoxin-damaged whole muscles has suggested that proliferative myoblasts rapidly congregate at the focus of injury, strongly implicating them as the subsequent regenerative source (Klein-Ogus & Harris 1983). However, isolated myoblasts have only a limited ability to contribute to myotoxin-induced regeneration after grafting into a new host muscle (Gross & Morgan 1999). Similarly, though grafted bone marrow-derived cells can regenerate skeletal muscle following myotoxin injury, their contribution is very low, and regenerated myofibres are distributed diffusely in the host muscle (Camargo *et al.* 2003, Corbel *et al.* 2003), providing little evidence of organised clonal expansion. Therefore although the regenerative behaviour of whole muscles strongly suggests the existence of a stem cell-like phenotype, the implied functional potential has not been localised to any adult cell isolate of either muscle or non-muscle origin.

5.12 Aims of work

Previous observations (see Chapter 4) showed that a proportion of single myofibre grafts give rise to satellite cells, and, under different experimental conditions, the majority of EDL myofibres grafts give rise to MPC that are subsequently proliferation-competent *in vitro*. The work described in this chapter aimed to investigate the response of such graft-derived cells to subsequent muscle damage. A notexin-induced injury model was used to investigate the ability of satellite cells and their progeny to respond to severe acute damage of the irradiated or non-irradiated graft site. *In vitro* experiments were used to investigate the response of satellite cells to damage in the absence of other components of the regenerative milieu.

5.13 Note on identification of regenerate graft products

To investigate the response of satellite cells to damage, myofibre grafts were derived from the muscles of *Myf5^{nLacZ/+}* mice, which express Myf5- β -gal fusion protein in the nuclei of satellite cells and newly-regenerated myofibres (Tajbakhsh *et al.* 1996a, Beauchamp *et al.* 2000) (2.14). Single myofibre-engrafted muscles were damaged by injection of the potent myotoxin, notexin (reviewed, Harris 2003). Newly-regenerated myofibres were identified both morphologically, by their small size and central nucleation (Coulton *et al.* 1988, Gross & Morgan 1999), and phenotypically, by the expression of neonatal myosin (Gross & Morgan 1999, Heslop *et al.* 2000) and myonuclear Myf5- β -gal fusion protein (Tajbakhsh *et al.* 1996a, Beauchamp *et al.* 2000, Zammit *et al.* 2004b).

In wild-type mice, neonatal myosin is expressed by a large proportion of myofibres in soleus, EDL, and gastrocnemius muscles during the early neonatal period of muscle maturation, but is lost by 21 days of age (Agbulut *et al.* 2003). Whilst neonatal myosin is not present in the TA muscles of wild-type mice between aged 35-168 days, *mdx* TA muscles have been found to contain some neonatal myosin⁺ myofibres when examined at 42 days of age or 49 days of age, even after previous exposure to 18 Gy irradiation (Heslop *et al.* 2000). Expression of neonatal myosin in adult muscle is induced by experimental manipulations such as injection of myotoxic agents (Gross & Morgan 1999, Heslop *et al.* 2000) or whole muscle grafting (Yoshimura *et al.* 1998b) and remains strong for at least 7-10 days after injury (Gross & Morgan 1999).

Neonatal myosin is also found in mature myofibres that are inadequately innervated (Yoshimura *et al.* 1998a, 1998b). Similarly, Myf5- β -gal fusion protein is re-expressed by fast-fibre myonuclei in response to denervation both *in vivo* (Zammit *et al.* 2004b) and *in vitro* in isolated single myofibres (Zammit *et al.* 2002). However, newly-regenerated myofibres are distinguishable from mature non-innervated (or developmentally aberrant) myofibres by their small size and central nucleation (Gross & Morgan 1999).

5.2 The response of grafted satellite cells to damage

5.21 Aim

The objective of these experiments was to investigate whether cells derived from single myofibre grafts have the ability to upregulate myogenic activity in response to subsequent severe acute damage of the engrafted muscle.

5.22 The effect of notexin on control *Myf5^{nLacZ/+}* muscles

As a control experiment, the effect of notexin on adult *Myf5^{nLacZ/+}* muscles was examined.

Both TA muscles of two 90-day-old male *Myf5^{nLacZ/+}* mice were damaged by injection of notexin (2.56). One mouse was sacrificed 24 h post-injection and the second mouse 7 d post-injection, and the damaged muscles harvested. The TA muscles of a third mouse functioned as non-damaged controls. Muscles were processed for immunohistochemistry (2.61) and four 7 μ m serial sections collected at 100 μ m intervals at four levels in each muscle (2.62). The first set of sections was stained in X-gal, (2.77), in the second set, fluorescence immunohistochemistry was used to detect expression of membrane-localised dystrophin and cytoplasmic neonatal myosin proteins (2.76), and the third set was stained with H & E (2.78). In non-damaged control muscles (Fig 5.1), only occasional nuclei expressing Myf5- β -gal fusion protein were seen, which were presumably those of satellite cells. As expected, all myofibres expressed dystrophin protein. Some myofibres were weakly stained by the neonatal myosin antibody. This may have been an artefact attributable to myofibre-specific endogenous autofluorescence (Jackson *et al.* 2004), or could have been due to

the recognised weak cross-reactivity of the BF34 antibody (**Table 2.1**) with slow myosin (Borrione *et al.* 1988). H & E stained control sections contained large, peripherally-nucleated myofibres. 24 h after notexin injection (**Fig. 5.1**), increased numbers of β -gal⁺ nuclei were observed in sections, probably due to myonuclear re-activation of Myf5- β -gal fusion protein (Zammit *et al.* 2002, 2004b). Dystrophin expression was weak or absent, as expected since structural proteins are reportedly degraded with 9 h of exposure to notexin (Harris *et al.* 2003). Some damaged myofibres appeared to stain positively for neonatal myosin, but the high potential for background in these acutely necrotic and disorganised muscles makes this observation unreliable. In H & E stained sections, the disorganisation of muscle tissue 24 h post-notexin injection was particularly apparent. Most myofibres had completely disintegrated, whilst others had become swollen, suggesting a lack of membrane integrity. An area of about 1/5 of the muscle appeared to escape injury in each case. In muscles removed 7 d after notexin injection (**Fig. 5.1**), the tissue had regenerated efficiently after injury. Some myofibres at the edge of muscles had escaped notexin damage, and were identifiable by their large size, peripheral nucleation and lack of expression of neonatal myosin or Myf5- β -gal fusion protein. Newly-regenerated myofibres were identifiable by their small size, central nucleation, and robust expression of neonatal myosin and Myf5- β -gal fusion protein. 7 d post-notexin injury, dystrophin expression was also restored, in agreement with the findings of a previous study (Gross & Morgan 1999).

In *mdx*-nude TA muscles engrafted with *Myf5^{nLacZ/+}* single myofibres and subsequently damaged, there were two potential phenotypic markers of donor origin, β -gal and dystrophin. Within areas of grafted-derived muscle, recently regenerated myofibres could be identified by expression of myonuclear β -gal and cytoplasmic neonatal myosin proteins, and also by their small size and central nucleation.

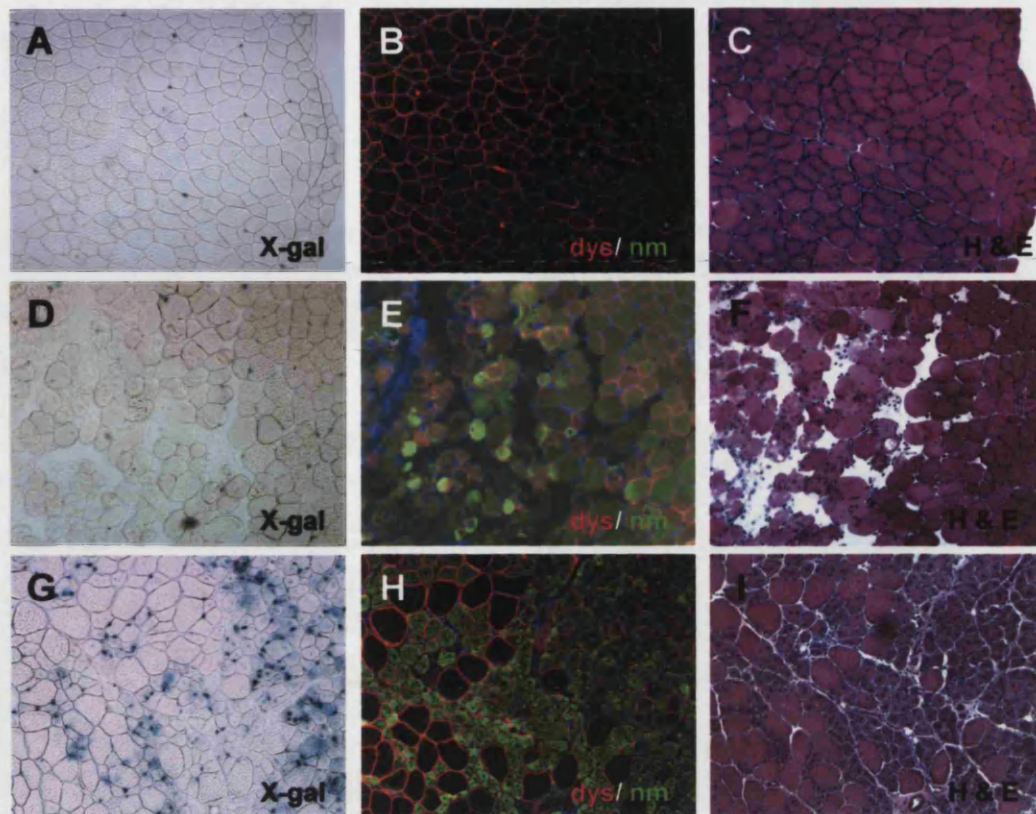


Fig. 5.1. Regeneration of *Myf5^{nLacZ/+}* TA muscles following notexin-induced injury. Serial sections: stained with X-gal (A, D, G), fluorescence immunohistochemistry for dystrophin and neonatal myosin (B, E, H), and stained with H & E (C, F, I). In a control muscle (A-C) *Myf5*- β -gal fusion protein is restricted to satellite cells (A), dystrophin expression is strong and neonatal myosin expression weak (B) and myofibers are large and peripherally nucleated (C). 24 h post-injury (D-F) β -gal⁺ nuclei are more frequent (D), the tissue has become necrotic and disorganised (E, F) and dystrophin protein has been degraded (E). Apparent expression of neonatal myosin (E) may be artefactual. 7 d post-injury (G-I) efficient regeneration has occurred, and dystrophin protein is restored (H). Myofibers which survived injury are identifiable by their lack of β -gal activity (G) or neonatal myosin (H), and by their large size and peripheral nucleation (I). Newly-regenerated myofibers are identifiable by their expression of neonatal myosin (H) myonuclear *Myf5*- β -gal fusion protein (G) and by their small size and central nucleation (I). Original magnification x100.

5.23 The effect of notexin on *Myf5^{nLacZ/+}* EDL single myofibre grafts

Single myofibres were isolated from the EDL muscles of a 42-day-old *Myf5^{nLacZ/+}* mouse and prepared for grafting as previously described (2.21, 3.42). Grafts were inserted (2.55, 3.42) into the pre-irradiated (2.53) TA muscles (n=34) of 17 *mdx*-nude host mice. Four weeks after grafting, 12/17 mice were re-anaesthetised (2.51) and muscle damage induced to bilateral engrafted TA muscles by injection of 10 μ l notexin (2.56). The 10 engrafted muscles of the remaining 5/17 mice served as non-damaged controls. Engrafted TA muscles were removed 5 weeks after grafting and 1 week after notexin damage, and processed for immunohistochemistry (2.61). Four serial sections were collected at 100 μ m intervals throughout the entire muscle body (2.62). The first set of slides were stained in X-gal (2.77). In muscles with detectable β -gal activity, in the second set of slides fluorescence immunohistochemistry was used to detect expression of dystrophin and neonatal myosin proteins (2.76), and the third set stained with H & E (2.78). The maximum numbers of total dystrophin⁺ myofibres, dystrophin⁺ neonatal myosin⁺ myofibres, and β -gal⁺ nuclei were counted in single serial sections for each muscle. In these experiments, counts were made of the total numbers of β -gal⁺ nuclei, rather than the total numbers of myofibres containing β -gal⁺ nuclei. Counts of β -gal⁺ nuclei therefore included both myonuclei, and presumptive satellite cells and MPC outside myofibres.

18/24 (75%) of engrafted muscles damaged with notexin, and 4/10 (40%) of undamaged engrafted control muscles contained both β -gal activity and dystrophin expression.

In the 4 successfully-engrafted undamaged control muscles, there were 114.0 (\pm 57.1) dystrophin⁺ myofibres of which 2.3 (\pm 2.2) co-expressed neonatal myosin, and 5.0 (\pm 4.7) nuclei expressing Myf5- β -gal fusion protein, positioned on the periphery of dystrophin⁺ myofibres. Their location and low frequency, combined with the lack of neonatal myosin expression and morphological markers of regeneration, suggests that these cells may have been satellite cells (Figs. 5.2 & 5.3).

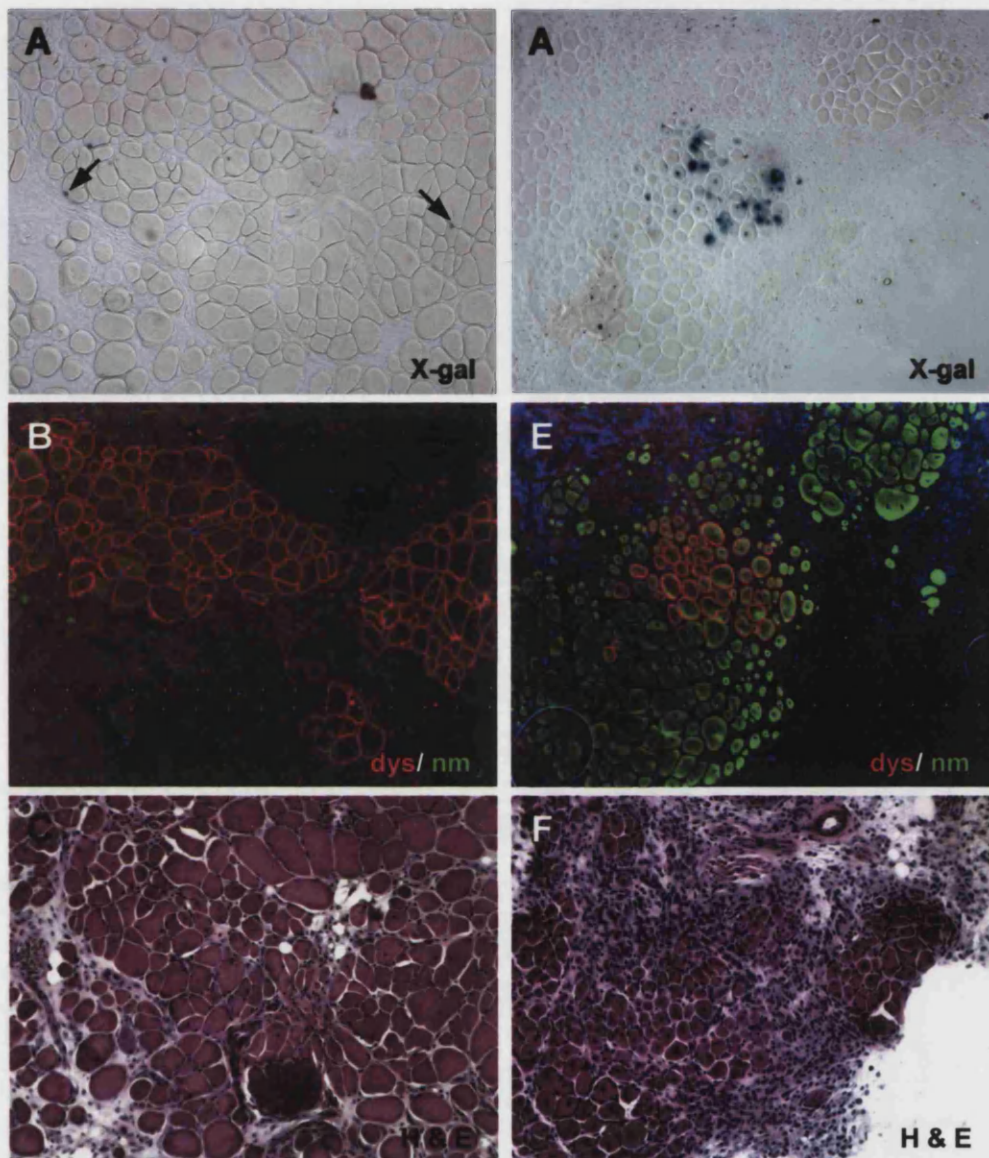


Fig. 5.2. Regeneration of *mdx*-nude TA muscles 5 weeks after engraftment with a single *Myf5^{nLacZ/+}* EDL myofibre, and 1 week after notexin-induced damage. Serial sections: stained with X-gal (A, D), immunohistochemistry for dystrophin and neonatal myosin (B, E), and stained with H & E (C, F). In an undamaged control muscle (A-C), only a few, peripheral β -gal⁺ (possibly satellite cell) nuclei are localised (A) (arrows). Dystrophin⁺ myofibres are neonatal myosin⁻ (B) and central nucleation is not pronounced. In notexin-damaged muscles (D-F), recently-regenerated myofibres of donor origin are identifiable by co-expression of myonuclear β -gal (D) and dystrophin and neonatal myosin (E), and by their small size and central nucleation (F). Original magnification x100.

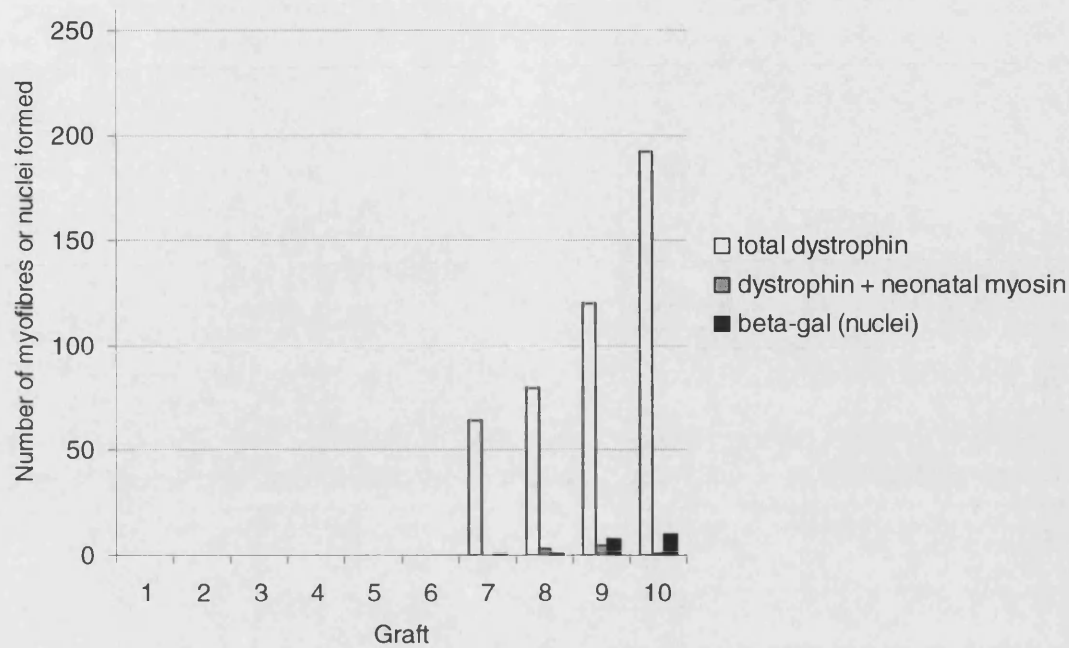


Fig. 5.3. Histogram showing muscle formation by individual *Myf5*^{nLacZ/+} EDL single myofibres 5 weeks after grafting. Ranked in order of the number of dystrophin⁺ myofibres. 4/10 (40%) of engrafted muscles contained β -gal activity and dystrophin⁺ myofibres. Very few dystrophin⁺ myofibres co-expressed neonatal myosin, a marker of recent regeneration. Associated with each group of dystrophin⁺ myofibres were occasional peripheral nuclei expressing Myf5- β -gal fusion protein. Their small numbers, the low level of neonatal myosin expression and the lack of morphological markers of recent regeneration (**Fig. 5.2**) suggest that these nuclei may have been those of satellite cells. Engrafted muscles with no detectable β -gal activity were not stained for dystrophin or neonatal myosin.

In the 18 successfully-engrafted notexin-damaged muscles, there were 70.0 (± 40.0) dystrophin⁺ myofibres of which 60.3 (± 40.1) strongly co-expressed neonatal myosin, and 30.0 (± 21.7) β -gal nuclei, many of them located in the centre of dystrophin⁺ myofibres. Myofibres expressing phenotypic markers of regeneration were usually morphologically small, and always centrally nucleated. Donor-derived myofibres with phenotypic and morphological markers of recent regeneration were located in single compact clusters, or infrequently in two or three clusters, suggesting that they had been formed by rapid clonal or oligoclonal expansion in the seven days following injury (**Fig. 5.2 & 5.4**). Although in most cases, more than 75% of each engrafted muscle appeared to have been severely damaged by the notexin treatment, diffuse distribution of donor-derived products was not seen.

Most engrafted notexin-damaged muscles also contained numerous myofibres that expressed neonatal myosin and morphological markers of recent regeneration, but contained no donor products (**Fig. 5.2**). These myofibres were presumed to be derived from endogenous radiation-resistant muscle stem cells, which have been shown previously to be myogenic in response to notexin injury (Heslop *et al.* 2000).

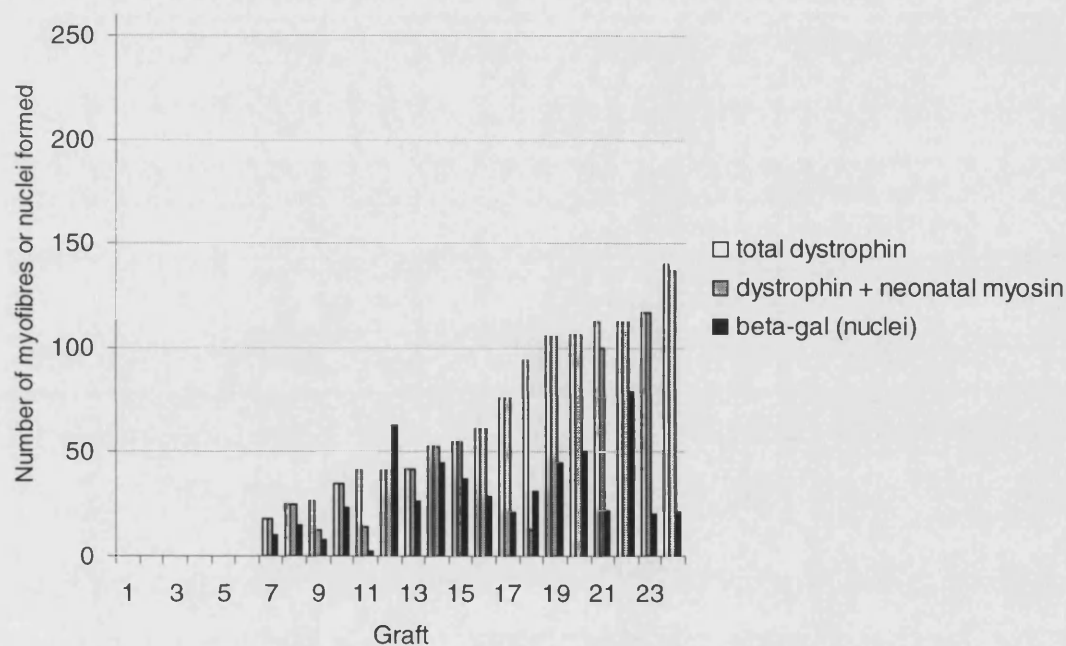


Fig. 5.4. Histogram showing newly-regenerated muscle derived from individual *Myf5^{nLacZ/+}* EDL single myofibre grafts in response to severe damage. Ranked in order of the number of dystrophin⁺ myofibres. 4 weeks after grafting, muscles were injured by injection of the myotoxin, notexin, and then removed for analysis 1 week later. 18/24 (75%) of engrafted muscles contained β -gal activity and dystrophin⁺ myofibres. In most grafts, the majority of dystrophin⁺ myofibres co-expressed neonatal myosin, and many also contained nuclei expressing Myf5- β -gal fusion protein, markers of recent regeneration. Counts of nuclei expressing Myf5- β -gal fusion protein include both myonuclei, and mononucleate MPC present outside myofibres. In comparison, grafts derived from the same animal, but not challenged with notexin, formed muscle in 40% of host sites and only expressed markers of regeneration in a few myofibres (**Figs. 5.2 and 5.3**). Engrafted muscles with no detectable β -gal activity were not examined for other markers.

There was no statistically significant difference (Mann-Whitney, $p > 0.05$) between the total numbers of dystrophin⁺ myofibres in undamaged and notexin-damaged engrafted muscles. Damaged muscles contained significantly higher numbers of dystrophin⁺ neonatal myosin⁺ myofibres (Mann-Whitney, $p = 0.0023$) and β -gal⁺ nuclei (Mann-Whitney, $p = 0.0053$) than undamaged muscles.

5.24 The effect of notexin on *Myf5*^{nLac/+} soleus single myofibre grafts

Single myofibres were isolated from the soleus muscles of a 42-day-old *Myf5*^{nLacZ/+} mouse and a 49-day-old *Myf5*^{nLacZ/+} mouse and prepared for grafting as previously described (2.21, 3.42). Grafts were inserted (2.55, 3.42) into the pre-irradiated (2.53) TA muscles ($n = 14$) of 7 *mdx*-nude host mice, split between two small experiments. Four weeks after grafting, mice were re-anaesthetised and notexin injected into the right engrafted TA muscles. The contralateral left engrafted TA muscles functioned as non-damaged controls. Engrafted TA muscles were removed 5 weeks after grafting and 1 week after notexin damage, and processed for immunohistochemistry (2.61). Four serial sections were collected at 100 μ m intervals throughout the entire muscle body (2.62). The first set of slides was stained in X-gal (2.77). The second set was stained for dystrophin and neonatal myosin proteins (2.76) and the third with H & E (2.78), as previously. The maximum numbers of total dystrophin⁺ myofibres, dystrophin⁺ neonatal myosin⁺ myofibres, and β -gal⁺ nuclei were counted in single serial sections for each muscle.

7/7 (100%) of notexin-damaged engrafted muscles and 7/7 (100%) of undamaged contralateral engrafted muscles contained both β -gal activity and dystrophin expression.

In undamaged muscles, there were 75.3 (± 56.8) dystrophin⁺ myofibres of which 31.0 (33.1) co-expressed neonatal myosin, and 3.3 (± 2.8) β -gal⁺ nuclei, most of which were located on the periphery of myofibres. In some control engrafted muscles, a large proportion of dystrophin⁺ myofibres co-expressed neonatal myosin and were centrally nucleated (Figs 5.5 & 5.6). This implies that muscle formation had continued to be augmented up until the five week timepoint.

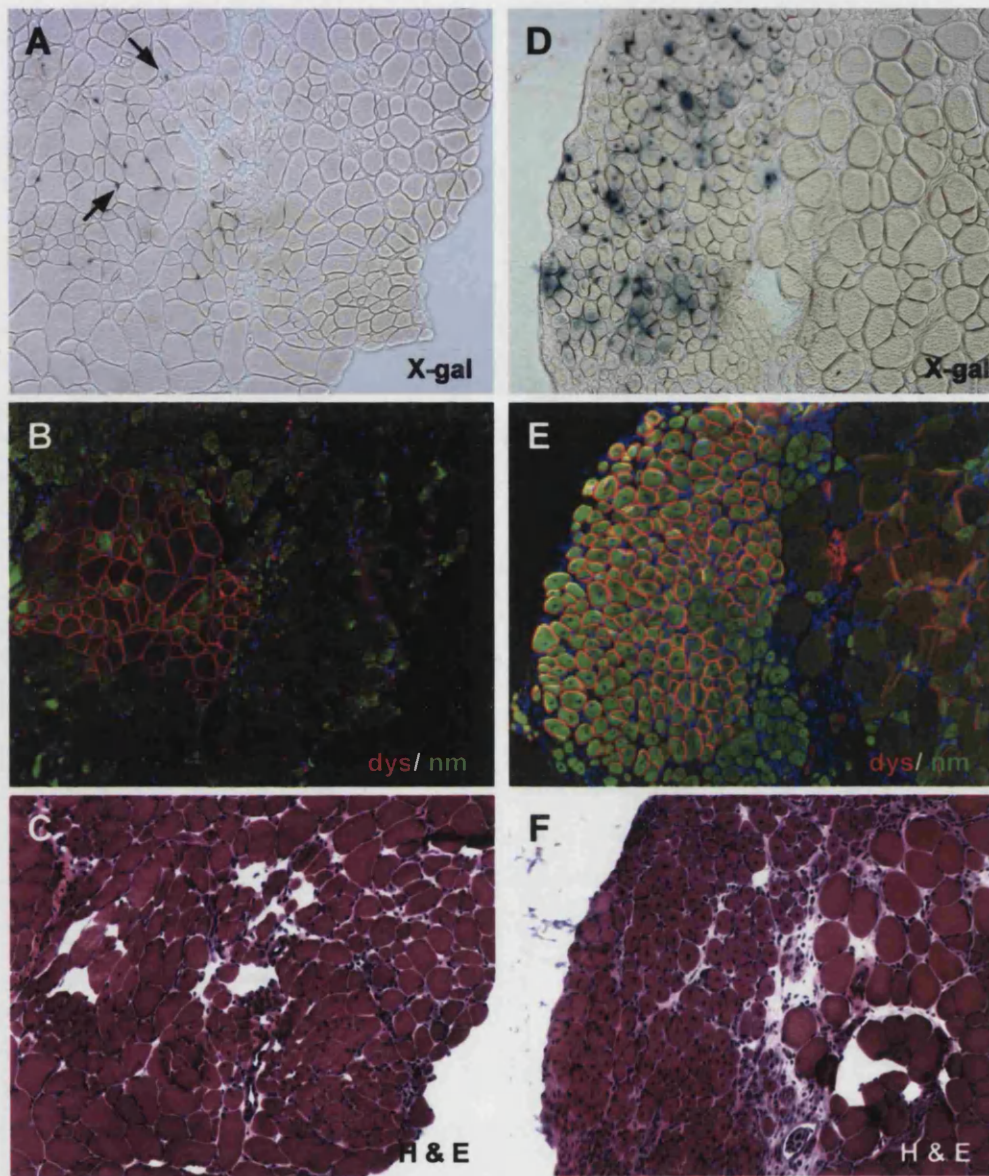


Fig. 5.5. Regeneration of *mdx*-nude TA muscles 5 weeks after engraftment with a single *Myf5^{nLacZ/+}* soleus myofibre, and 1 week after notexin-induced damage. Serial sections: stained with X-gal (A, D), immunohistochemistry for dystrophin and neonatal myosin (B, H), and stained with H & E (C, F). In an undamaged control muscle (A-C), several peripheral β -gal⁺ nuclei are localised, (A) (arrows). Some dystrophin⁺ myofibres express neonatal myosin (B) but central nucleation is not pronounced. In a contralateral notexin-damaged muscle (D-F), recently-regenerated myofibres of donor origin are identifiable by co-expression of myonuclear β -gal (D) and dystrophin and neonatal myosin (E), and by their small size and central nucleation (F). Original magnification x100.

In damaged muscles, there were $94.7 (\pm 74.3)$ dystrophin⁺ myofibres of which 93.1 (75.2) strongly co-expressed neonatal myosin, and $44.4 (\pm 50.1)$ β -gal⁺ nuclei, many in the centre of dystrophin⁺ myofibres. As previously, most myofibres expressing phenotypic regenerative markers were morphologically small and centrally-nucleated, and formed compact clusters (**Figs. 5.5 & 5.7**).

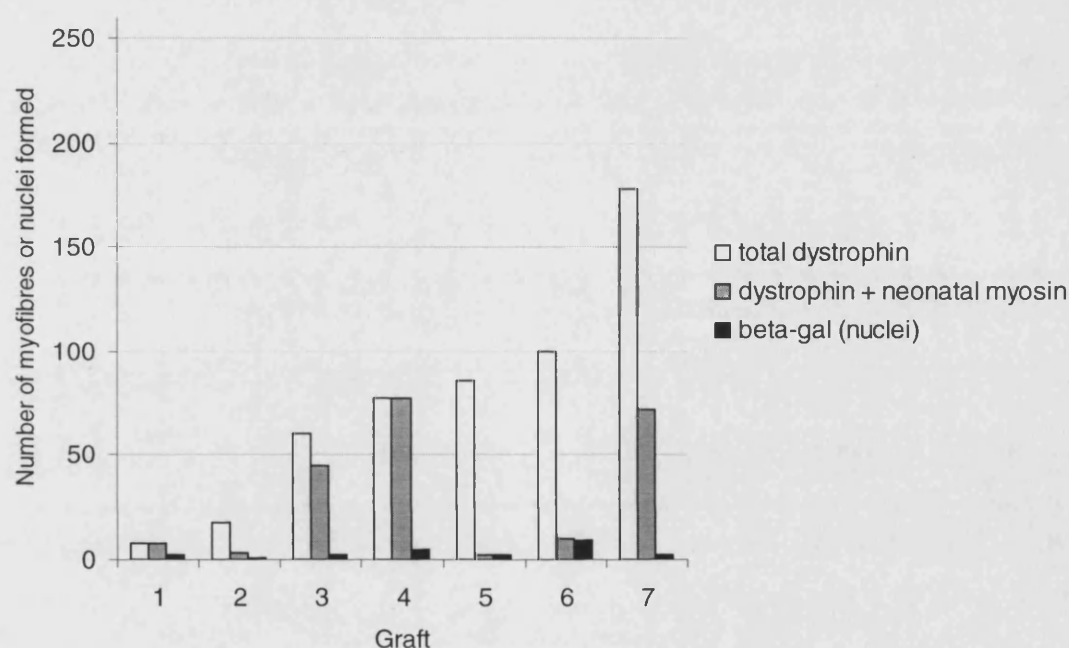


Fig. 5.6. Histogram showing muscle formation by individual grafts of *Myf5*^{nLacZ/+} soleus single myofibres 5 weeks after grafting. Ranked in order of the the number of dystrophin⁺ myofibres. The left engrafted TA muscle of each mouse (data shown here), was left undamaged, whilst the right contralateral leg was damaged with notexin (**Fig. 5.7**). Here, without damage, 100% (7/7) of engrafted muscles contained β -gal activity and dystrophin expression. In several engrafted muscles, although only a few myonuclei expressed *Myf5*- β -gal fusion protein, many dystrophin⁺ myofibres co-expressed neonatal myosin, a marker of recent regeneration, and were centrally nucleated (**Fig. 5.5**). Pooled data from two experiments.

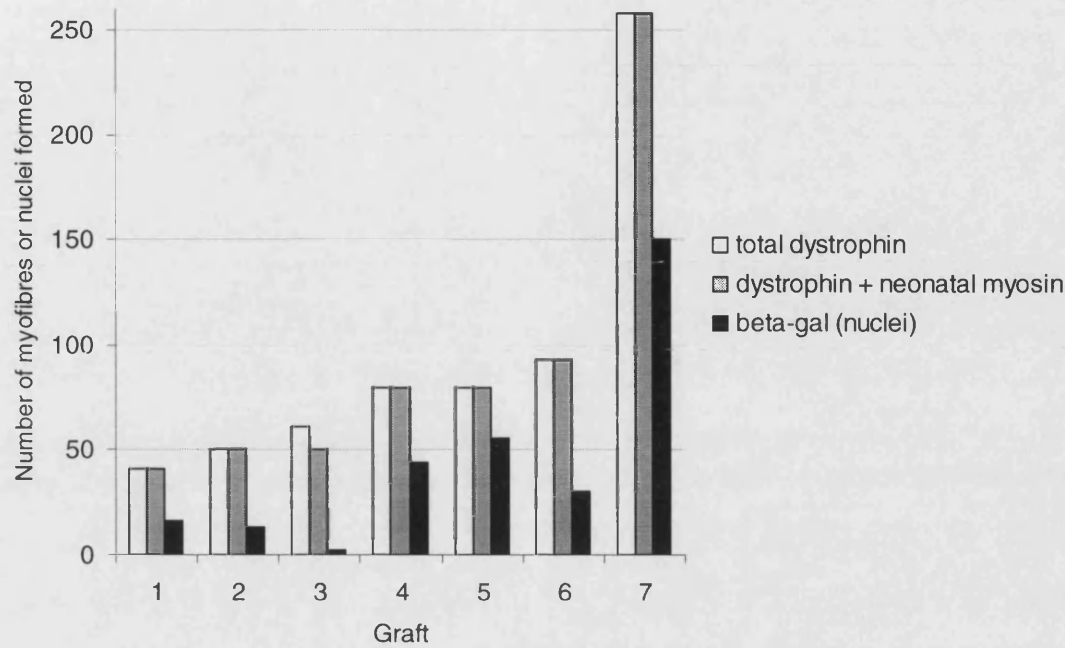


Fig. 5.7. Histogram showing newly-regenerated muscle derived from individual *Myf5^{nLacZ/+}* soleus single myofibre grafts in response to severe damage. Ranked in order of the number of dystrophin⁺ myofibres. The left engrafted TA muscle of each mouse was left undamaged, (**Fig. 5.6**), whilst the right contralateral leg was damaged by injection of notexin 1 week before removal (data shown here). 100% (7/7) of engrafted muscles contained β -gal activity and dystrophin expression. Most dystrophin⁺ myofibres co-expressed neonatal myosin, and many also contained myonuclei that expressed Myf5- β -gal fusion protein, markers of recent regeneration. Counts of nuclei expressing Myf5- β -gal fusion protein include both myonuclei, and mononucleate MPC present outside myofibres. Pooled data from two experiments.

There was no statistically-significant difference (Mann-Whitney, $p \geq 0.05$) between the total numbers of dystrophin⁺ myofibres in undamaged and notexin-damaged engrafted muscles. Damaged muscles contained significantly higher numbers of dystrophin⁺ neonatal myosin⁺ myofibres ($p=0.0262$) and β -gal⁺ nuclei ($p=0.0070$) than undamaged muscles.

5.25 The effect of notexin on *Myf5^{nLac/+}* TA single myofibre grafts

Single myofibres were isolated from the TA muscles of a 42-day-old *Myf5^{nLacZ/+}* mouse and a 49-day-old *Myf5^{nLacZ/+}* mouse and prepared for grafting as previously described (2.21, 3.42). The two donor mice were the same animals used for the soleus myofibre grafts (5.23). Grafts were inserted (2.55, 3.42) into the pre-irradiated (2.53) TA muscles (n=16) of 8 *mdx*-nude host mice, split between two small experiments. Four weeks after grafting, mice were re-anaesthetised and notexin injected into the right engrafted TA muscles, with the contralateral muscles functioning as controls. Engrafted TA muscles were removed 5 weeks after grafting and 1 week after notexin damage, and processed for immunohistochemistry (2.61). Four serial sections were collected at 100 μ m intervals throughout the entire muscle body (2.62). The first set of slides was stained in X-gal (2.77), the second set was stained for dystrophin and neonatal myosin proteins (2.76) and the third with H & E (2.78), as previously. The maximum numbers of total dystrophin⁺ myofibres, dystrophin⁺ neonatal myosin⁺ myofibres, and β -gal⁺ nuclei were counted in single serial sections for each muscle.

8/8 (100%) of notexin-damaged engrafted muscles and 5/8 (63%) of contralateral undamaged engrafted muscles contained both β -gal activity and dystrophin expression.

In successfully-grafted undamaged muscles (5/8), there were 85.6 (\pm 89.1) dystrophin⁺ myofibres of which 73.2 (\pm 89.4) co-expressed neonatal myosin, and 2.8 (\pm 1.8) β -gal⁺ nuclei, most of which were located on the periphery of myofibres (Figs 5.8 & 5.10). Two grafts (nos. 7 and 8 on graph, Fig. 5.10) formed more than 150 dystrophin⁺ myofibres, most of which co-expressed neonatal myosin, suggesting that they had been formed shortly before the five week timepoint. This was unexpected, since in previously described experiments, grafts of 3F-*nLacZ*-2E myofibres harvested at the three week timepoint usually formed no more than 11 donor-derived myofibres (3.4).

In damaged muscles, there were 37.1 (\pm 25.9) dystrophin⁺ myofibres of which 35.4 (\pm 27.4) co-expressed neonatal myosin, and 15.2 (\pm 11.2) β -gal⁺ nuclei, mostly in the centre of dystrophin⁺ myofibres (Figs. 5.8 & 5.9). There was no statistically-significant difference (Mann-Whitney, $p > 0.05$) between the total numbers of

dystrophin⁺ myofibres in control and notexin-damaged engrafted muscles. In contrast to equivalent experiments using grafts of EDL or soleus myofibres (5.23, 5.24), the difference between the number of dystrophin⁺ neonatal myosin⁺ myofibres in undamaged and damaged TA myofibre-engrafted muscles was not significant (Mann-Whitney, $p \geq 0.05$). This was due to the high numbers of dystrophin⁺ neonatal myosin⁺ myofibres that were present in the absence of damage. However, damaged muscles did contain significantly higher numbers of β -gal⁺ myofibres than undamaged muscles.

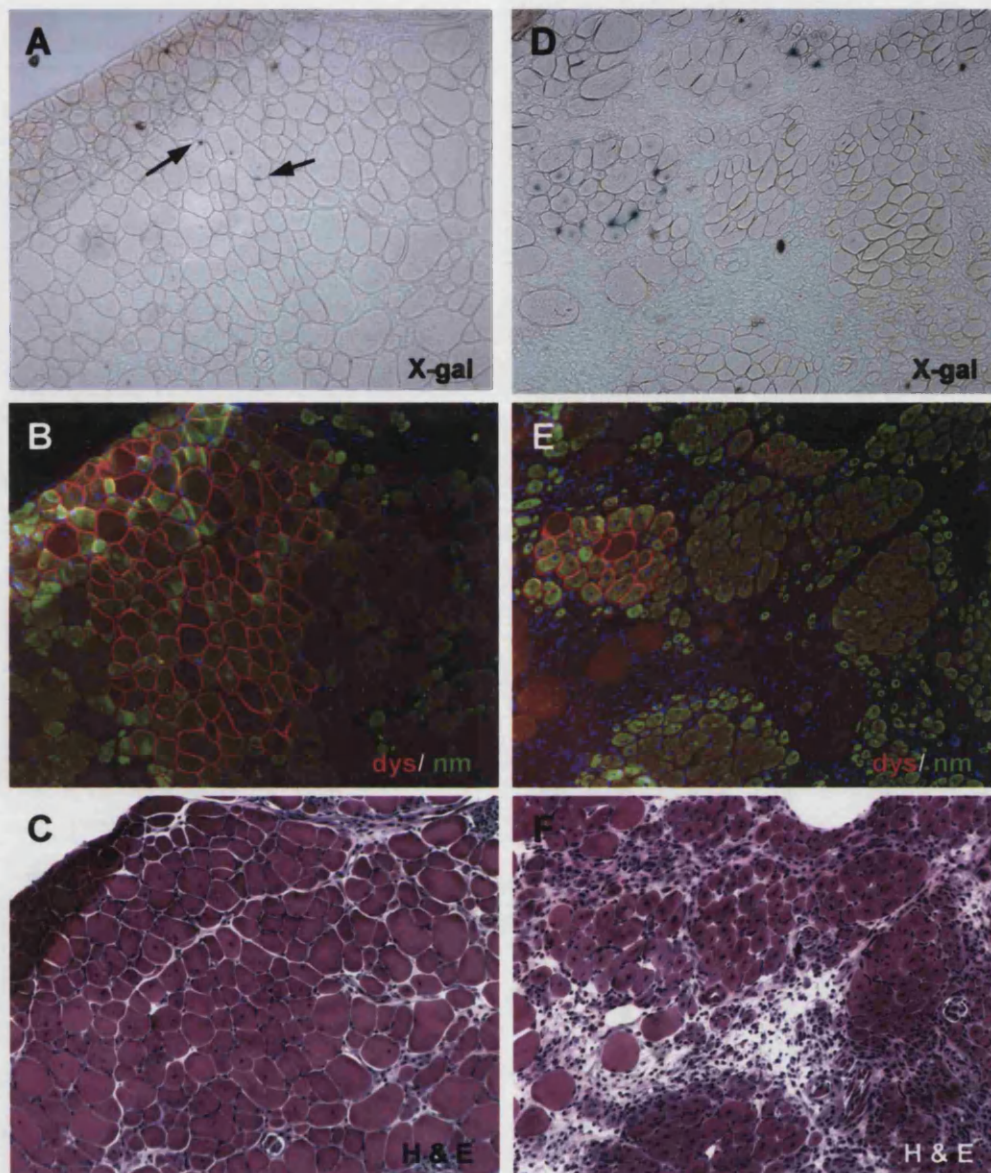


Fig. 5.8. Regeneration of *mdx*-nude TA muscles 5 weeks after engraftment with a single *Myf5^{nLacZ/+}* TA myofibre, and 1 week after notexin-induced damage. Serial sections: stained with X-gal (A, D), fluorescence immunohistochemistry for dystrophin and neonatal myosin (B, E), and stained with H & E (C, F). In an undamaged control muscle (A-C), a few, peripheral β -gal⁺ nuclei are localised (A) (arrows). Many dystrophin⁺ myofibres co-express neonatal myosin (B) and central nucleation is frequent. In notexin-damaged muscle (D-F), recently-regenerated myofibres of donor origin are identifiable by co-expression of myonuclear β -gal (D) and dystrophin and neonatal myosin (E), and by their small size and pronounced central nucleation (F). Original magnification x100.

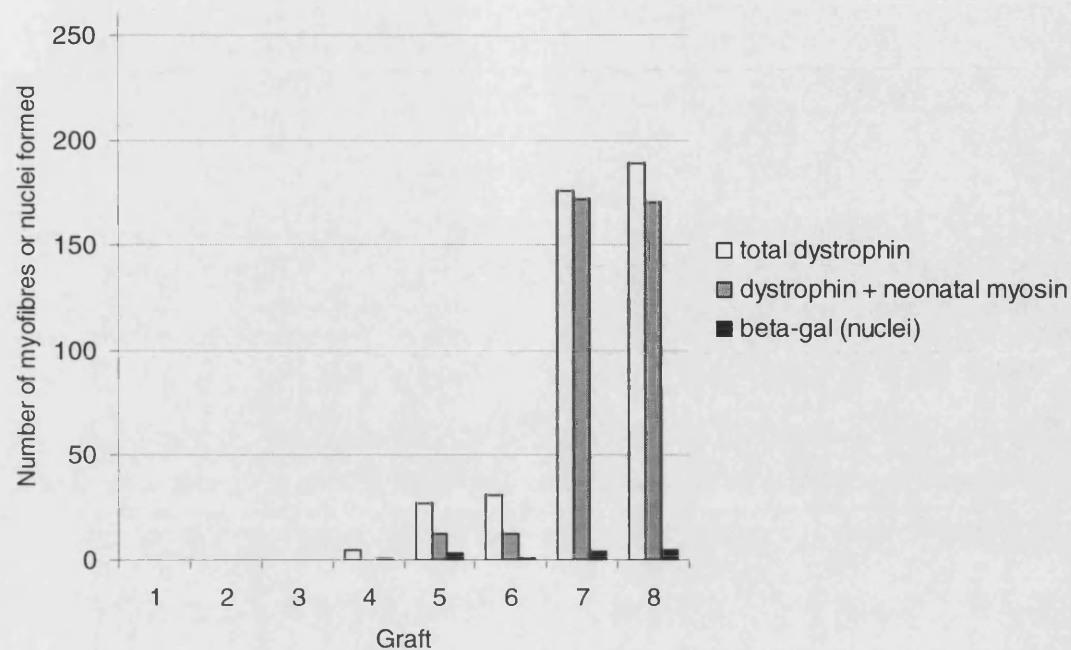


Fig. 5.9. Histogram showing muscle formation by individual grafts of *Myf5*^{nLacZ/+} TA single myofibres 5 weeks after grafting. Ranked in order of the the number of dystrophin⁺ myofibres. The left engrafted TA muscle of each mouse (data shown here), was left undamaged, whilst the right contralateral leg was damaged with notexin (**Fig. 5.10**). Here, without damage, 63% (5/8) of engrafted muscles contained β -gal activity and dystrophin expression. In 4/5 successfully engrafted muscles, although only a few myonuclei expressed *Myf5*- β -gal fusion protein, most dystrophin⁺ myofibres co-expressed neonatal myosin and were centrally nucleated (**Fig. 5.8**), suggesting that they had formed recently, in the absence of experimentally-induced damage. Pooled data from two experiments.

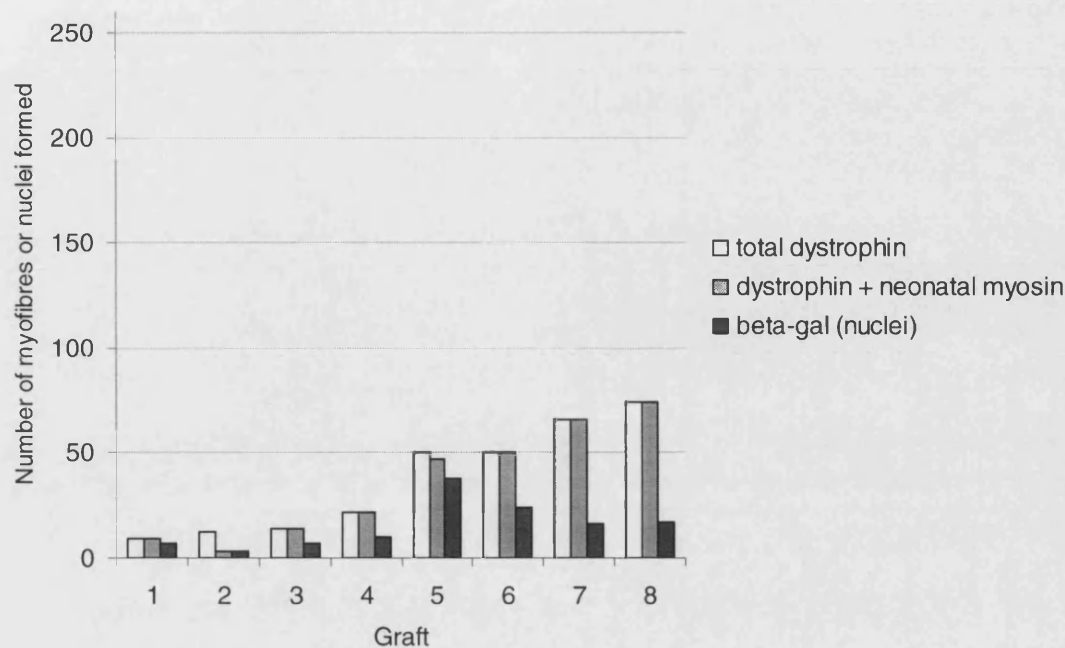


Fig. 5.10. Histogram showing newly-regenerated muscle derived from individual *Myf5*^{nLacZ/+} TA single myofibre grafts in response to severe damage. Ranked in order of the the number of dystrophin⁺ myofibres. The left engrafted TA muscle of each mouse was left undamaged, (**Fig. 5.9**), whilst the right contralateral leg was damaged by injection of notexin 1 week before removal (data shown here). 100% (8/8) of engrafted muscles contained β -gal activity and dystrophin expression. Most dystrophin⁺ myofibres co-expressed neonatal myosin, and many also contained myonuclei that expressed Myf5- β -gal fusion protein, markers of recent regeneration. Counts of nuclei expressing Myf5- β -gal fusion protein include both myonuclei, and mononucleate MPC present outside myofibres. Pooled data from two experiments.

5.26 The response of graft-derived cells to repeated bouts of damage

75% of grafted EDL myofibres and 100% of grafted soleus and TA myofibres were found to generate at least one cell capable of undergoing rapid clonal expansion in response to notexin damage (**5.22, 5.23, 5.24**). Since there are several putatively myogenic satellite cells associated with each myofibre (**3.41**), it was not clear from these experiments whether muscle was formed exclusively from cells that had previously never been activated, or whether it was derived from newly-formed donor-derived satellite cells that had been generated during an initial myogenic event. The aim of this experiment was to investigate whether graft-derived satellite cells were capable of regenerating tissue after a second round of acute damage, and therefore of maintaining their own population after the first regenerative event.

Single myofibres were isolated from the EDL muscles of a 43-day-old *Myf5^{enLacZ/+}* mouse and prepared for grafting as previously described (2.21, 3.42). Grafts were inserted (2.55, 3.42) into the pre-irradiated (2.53) TA muscles (n=16) of 8 *mdx*-nude host mice. Four weeks after grafting, mice were re-anaesthetised (2.51) and muscle damage induced to bilateral engrafted TA muscles by injection of notexin (2.56). Muscles were left to regenerate for three weeks, after which mice were again re-anaesthetised (2.51) and notexin injected into the right engrafted TA muscles. (2.56). The contralateral left TA muscles were not damaged a second time. Muscles were removed eight weeks after grafting, four weeks after the first round of notexin damage and one week after the second round of notexin damage, and processed for immunohistochemistry (2.61). Four serial sections were collected at 100 μ m intervals throughout the entire muscle body (2.62). The first set of slides were stained in X-gal (2.77), in the second set of slides, fluorescence immunohistochemistry was used to detect expression of dystrophin and neonatal myosin proteins (2.76), and the third set stained with H & E (2.78). Unfortunately, two of the left engrafted muscles (subjected to one round of damage only) had to be discarded. The maximum numbers of total dystrophin⁺ myofibres, dystrophin⁺ neonatal myosin⁺ myofibres, and β -gal⁺ nuclei were counted in single serial sections for each muscle.

5/8 (63%) of twice-damaged muscles and 3/6 (50%) of once-damaged muscles contained both β -gal activity and dystrophin expression. In the 5/8 successfully-grafted twice-damaged muscles, there were 45.8 (\pm 31.5) dystrophin⁺ myofibres of which 45.2 (\pm 32.1) co-expressed neonatal myosin, and 31.0 (\pm 28.1) β -gal⁺ nuclei, mainly within dystrophin⁺ myofibres. Donor-derived myofibres formed small, centrally-nucleated clusters (Figs. 5.11 & 5.12). After two bouts of notexin damage, the host regenerative response appeared to be less efficient than after one bout of damage; however, host-derived regenerating myofibres were not quantified. Clusters of donor-derived myofibres were less compact than those generated after a single bout of damage, but nonetheless, diffuse distribution was not observed. As in previous experiments, it is therefore likely that muscle was generated from closely associated cells, or possibly from a single cell.

In the 3/6 successfully-grafted once-damaged contralateral host TA muscles, there were 135.7 (± 153.8) dystrophin⁺ myofibres of which 105.0 (± 180.8) co-expressed neonatal myosin, and 45.3 (± 64.7) β -gal⁺ nuclei. Two grafts formed small amounts of muscle with little expression of regenerative markers, whereas one graft formed an exceptionally large amount of muscle (313 dystrophin⁺ myofibres), with high co-expression of molecular markers of regeneration, and frequent central nucleation (**Fig. 5.13**). These data suggest that in at least some grafts, the regenerative response can persist until at least 4 weeks post-notexin damage.

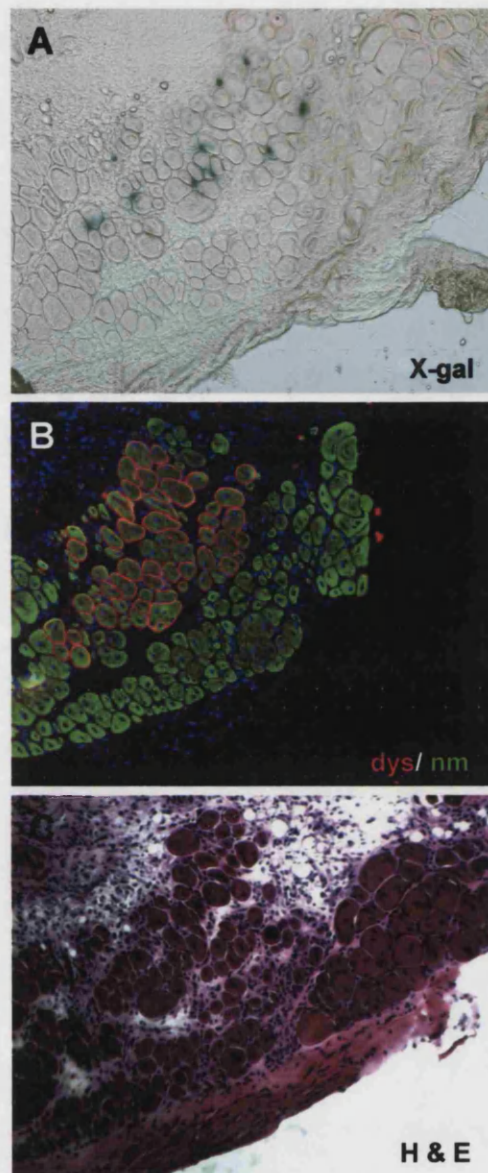


Fig. 5.11. Regeneration of an *mdx*-nude TA muscle 8 weeks after engraftment with a single *Myf5^{nLacZ/+}* EDL myofibre, and 1 week after two serial rounds of notexin-induced damage at 4 weeks and 7 weeks after grafting. Serial sections: stained in X-gal (A), fluorescence immunohistochemistry for dystrophin and neonatal myosin (B), and stained with H & E (C). Newly-regenerated donor-derived myofibres are identifiable by co-expression of Myf5-β-gal fusion protein (A) and dystrophin and neonatal myosin (B) and by their small size and central nucleation (C). Original magnification x100.

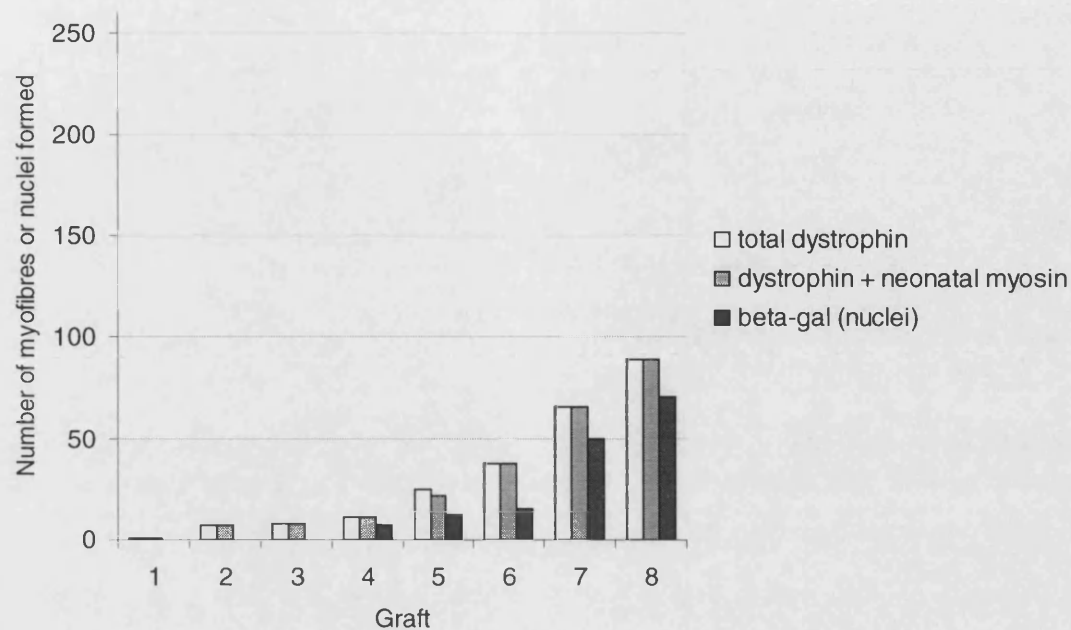


Fig. 5.12. Histogram showing newly-regenerated muscle derived from individual *Myf5^{nLacZ/+}* EDL single myofibre grafts after two rounds of severe damage. Ranked in order of the number of dystrophin⁺ myofibres. 4 weeks after grafting, both the left and right engrafted TA muscles were injured by injection of the myotoxin, notexin. After a further 3 weeks, right engrafted muscles were further injured with a second notexin injection (shown here). Contralateral left engrafted TA muscles were not re-injured (**Fig. 5.13**). Here, engrafted muscles were removed 8 weeks after grafting, 4 weeks after the first notexin injection and 1 week after the second notexin injection. Counts of nuclei expressing Myf5- β -gal fusion protein include both myonuclei, and mononucleate MPC present outside myofibres. In all successful grafts, most dystrophin⁺ myofibres co-expressed neonatal myosin and many co-expressed Myf5- β -gal fusion protein, showing that grafted satellite cells retained the ability to respond to a second round of damage (**Fig. 5.11**).

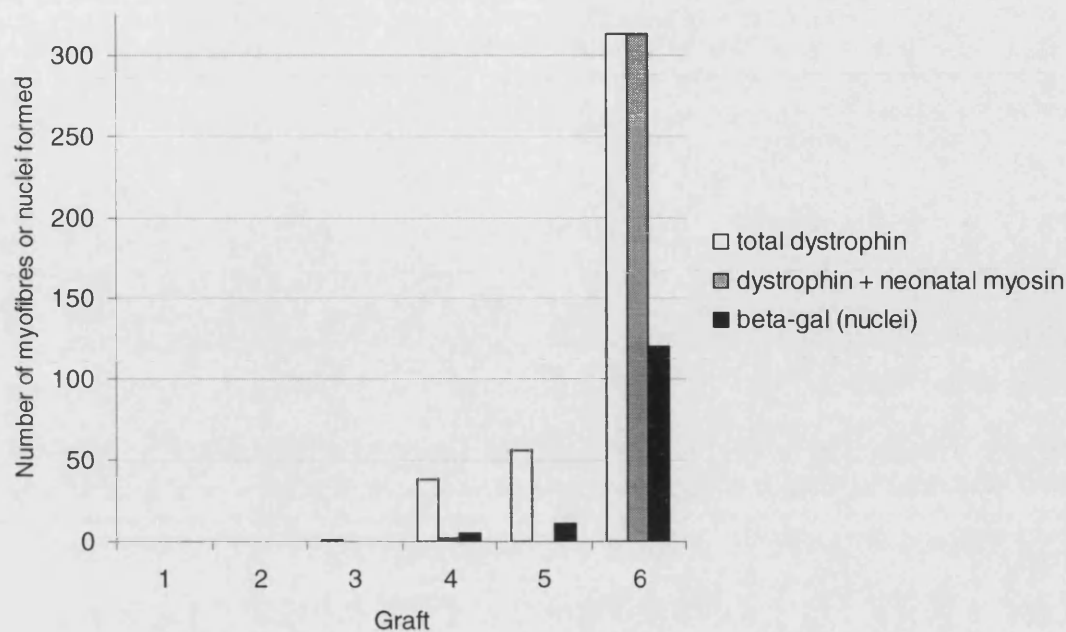


Fig. 5.13. Histogram showing newly-regenerated muscle derived from individual *Myf5*^{nLacZ/+} EDL single myofibre grafts 4 weeks after severe damage. Ranked in order of the number of dystrophin⁺ myofibres. 4 weeks after grafting, both the left and right engrafted TA muscles were injured by injection of notexin. After a further 3 weeks, right engrafted muscles were further injured with a second notexin injection (**Fig. 5.12**). Contralateral left engrafted TA muscles were not re-injured (shown here). These muscles were removed 8 weeks after grafting and 4 weeks after the single notexin injection. 3/6 (50%) of engrafted muscles contained β -gal activity and dystrophin⁺ myofibres. Counts of nuclei expressing Myf5- β -gal fusion protein include both myonuclei, and mononucleate MPC present outside myofibres. 1 successfully-engrafted muscles contained 313 dystrophin⁺ neonatal myosin⁺ myofibres and 120 β -gal⁺ nuclei, showing that the regenerative response had persisted up to 4 weeks post-damage.

	EDL myofibre			soleus myofibre		TA myofibre	
No. satellite cells per graft	7 ^a			22 ^a		10 ^a	
Treatment	undamaged	notexin	2 nd notexin	undamaged	notexin	undamaged	notexin
No. of grafts	10	24	8	7	7	8	8
Percentage of successful grafts ^b	40%	75%	62.5%	100%	100%	62.5%	100%
Total dys ⁺ myofibres formed by all grafts	456	1259	229	527	663	453	297
Total dys ⁺ nm ⁺ myofibres formed by all grafts	9	984	226	217	652	366	283
Total β -gal ⁺ nuclei formed by all grafts	20	547	155	23	311	14	124
Overall efficiency of all grafts ^c	6.51	7.49	4.09	3.42	4.31	5.66	3.71
Max. dys ⁺ myofibres from a single graft	192	140	89	178	258	189	66
Max. dys ⁺ nm ⁺ myofibres from a single graft	5	137	89	72	258	172	66
Max. β -gal ⁺ nuclei from a single graft	10	79	71	9	150	5	38
Max. efficiency of a single graft ^c	27.42	20.00	12.70	8.09	11.72	18.90	6.60

^a Mean Pax7⁺ satellite cells per single myofibre (**3.41**). ^b Grafts which generated ≥ 1 β -gal⁺ nuclei. ^c Efficiency= the total dystrophin⁺ myofibres formed divided by the total myogenic cells grafted. dys= dystrophin. nm= neonatal myosin.

Table. 5.1. Comparison of the efficiency of muscle regeneration derived from single myofibre grafts 1 week after notexin-induced damage.

5.27 Summary: comparison of the response of single myofibre grafts to damage

(a) Comparison of regeneration in control engrafted muscles harvested at the 5 week timepoint

The Kruskal-Wallis test was used to compare expression of dystrophin and of regenerative markers between muscle formed by grafts of EDL, soleus and TA myofibres harvested at the 5 week timepoint without notexin-induced damage (**Table 5.2**). There was no statistically significant difference between the numbers of total dystrophin⁺ myofibres formed from grafts derived from the three different muscles ($p > 0.05$). However, the difference between the number of myofibres co-expressing dystrophin and neonatal myosin was significant between the three muscles ($p = 0.0171$). In addition, the difference between the numbers of β -gal⁺ nuclei was very significant ($p = 0.0022$). The efficiency of muscle formation by control grafts was 6.51 for EDL myofibres, 3.42 for soleus myofibres, and 5.66 for TA myofibres (**Table 5.1**).

When in previous experiments, engrafted muscles were analysed at the 3 week timepoint, single myofibres formed muscle with lower frequency, and lower efficiency than at the 5 week timepoint, and the difference between the numbers of myofibres formed by grafts derived from the three different muscles was extremely significant (**3.46, Table 3.6**). Most notably, at the 3 week timepoint the efficiency of muscle formation by TA myofibre grafts was more than ten times less than that of EDL or soleus myofibre grafts (**Table 3.6**), but by the 5 week timepoint, efficiency of muscle formation by TA myofibre grafts was comparable to that of EDL myofibre grafts and greater to that of soleus myofibre grafts (**Table 5.1**). These differences are too substantial to be exclusively explained by potential inter-animal variability of donors, and strongly suggest that muscle formation had spontaneously augmented up until the 5 week timepoint.

	EDL single myofibre	soleus single myofibre	TA single myofibre
Satellite cells per graft ^a	7	22	10
No. of grafts	10	7	8
Percentage of successful ^b grafts	40.0%	100%	62.5%
Mean dystrophin ⁺ myofibres formed by successful grafts	114.0 (±57.1)	75.29 (±56.8)	85.60(±89.1)
Mean dystrophin ⁺ neonatal myosin ⁺ myofibres formed by successful grafts	2.3 (±2.2)	31.0 (±33.1)	73.20 (±89.4)
Mean β -gal ⁺ nuclei formed by successful grafts	5.00 (±4.7)	3.29 (±2.8)	2.80 (±1.8)

^a Mean Pax7⁺ satellite cells per single myofibre (3.41). ^b Grafts with ≥ 1 β -gal⁺ nuclei.

Table 5.2. Comparison of the quantities of muscle formed by control grafts of *Myf5^{nLacZ/+}* single myofibres harvested at the 5 week timepoint.

(b) Comparison of regeneration in engrafted muscles 1 week after notexin damage

The results of the experiments described in 5.22, 5.23 and 5.24 showed that satellite cells derived from the EDL, soleus and TA muscles of young adult mice have the ability to undergo rapid clonal expansion in response to damage. The Kruskal-Wallis test was used to compare notexin-induced regeneration generated by myofibres derived from EDL, soleus and TA muscles (Table 5.3). There were no statistically significant differences between the numbers of dystrophin⁺ myofibres, or between the numbers of dystrophin⁺ neonatal myosin⁺ myofibres formed by single myofibre grafts derived from the three different muscles ($p > 0.05$). The lack statistical significance may have been due to small sample number. However, there was a statistically significant difference ($p < 0.05$) between the numbers of β -gal⁺ nuclei formed by myofibre grafts derived from different muscles.

The efficiencies with which grafted single myofibres regenerated new myofibres after damage differed according to their muscle of origin (**Tables 5.1 and 5.2**). Soleus (**5.23**) and TA (**5.24**) single myofibre grafts were isolated from the muscles of the same two donor *Myf5^{nLacZ/+}* mice, allowing the regenerative potential of the satellite cell populations of the two muscles to be compared in the absence of artefacts generated by inter-animal variability. In response to notexin-induced damage, the mean number of dystrophin⁺ myofibres formed by TA myofibre grafts was only 40% of the mean number formed by soleus myofibre grafts derived from the same donor animals (**5.23**). However, the overall efficiencies of muscle formation were quite similar, at 4.31 for soleus myofibre grafts and 3.71 for TA myofibre grafts (**Table 5.1**). At 7.49, the overall efficiency of damage-induced regeneration generated from EDL myofibre grafts (derived from a different donor) (**5.21**) was higher than that of either soleus or TA myofibre grafts. In response to a second bout of damage (**5.25**) new myofibres were regenerated by EDL myofibre grafts with a reduced efficiency of 4.09. This difference, however, is probably not great enough to exclude the possibility that it is partly attributable inter-animal variability of donors. After two bouts of damage (**5.25**) EDL satellite cells generated new muscle with comparable efficiency to single myofibres derived from soleus or TA muscles after one bout of damage (**Table 5.1**).

	EDL single myofibre	EDL single myofibre, 2 nd round	soleus single myofibre	TA single myofibre
Satellite cells per graft ^a	7	7	22	10
No. of grafts	24	8	7	8
Percentage of successful ^b grafts	75.0%	62.5%	100%	100%
Mean dystrophin ⁺ myofibres formed by successful grafts	70.0 (±40.0)	45.80 (±31.5)	94.71 (±74.3)	37.12 (±25.9)
Mean dystrophin ⁺ neonatal myosin ⁺ myofibres formed by successful grafts	63.0 (±40.1)	45.20 (±32.1)	93.14 (±75.2)	35.38 (±27.4)
Mean β-gal ⁺ nuclei formed by successful grafts	30.0 (±21.7)	31.00 (±28.1)	44.43 (±50.1)	15.50 (±11.2)

^a Mean Pax7⁺ satellite cells per single myofibre (3.41). ^b Grafts with ≥1 β-gal⁺ nuclei.

Table 5.3. Comparison of the quantities of muscle formed by grafts of *Myf5*^{nLacZ/+} single myofibres 1 week after notexin-induced damage.

5.3 The effect of notexin on single myofibre cultures

5.31 Aim

The results of the experiments previously described in this chapter showed that satellite cells are the origin of efficient muscle regeneration after notexin damage (**Table 5.1**). In principle, myogenic activity could have been stimulated either by the damage induced by notexin, or by the notexin itself. The objective of this experiment was to use single myofibre cultures to investigate the effects of notexin on myofibre viability and satellite cell proliferation *in vitro*, outside the regenerative milieu of damaged muscle.

5.32 Exposure of single myofibre cultures to notexin

Single myofibres were isolated from the soleus muscles of a 43-day-old male C57 Bl/10 mouse, and cultured individually in the wells of 24-well-plates (**2.21**). After 48 h *in vitro*, counts were made of the number of cells surrounding each myofibre. Notexin (1 ng/ml, 10 ng/ml, 100ng/ml, 1 µg/ml or 10 µg/ml) was added to the plating medium of each culture, and left overnight. Ten replicates of each concentration were carried out, and in addition, ten control cultures without notexin. After 12 h, notexin was removed, cultures were gently washed with warmed PBS, and fresh warmed plating medium added. Cells were cultured for a further 72 h and the number of cells surrounding each myofibre counted at 24 h intervals. 120 h after plating and 72 h after notexin exposure, cultures were fixed (**2.71**) and desmin immunocytochemistry (**2.74**) used to verify the myogenic lineage of emanating cells (Bockhold *et al.* 1998). 2 of a total of 60 cultures contained small numbers of desmin⁻ cells.

Notexin concentrations in the range 100 ng/ml to 10 µg/ml caused 100% of myofibres to hypercontract (**Fig. 5.14**). Only 1 myofibre survived exposure to 10 ng/ml notexin. All myofibres exposed to 1 ng/ml and all controls survived. Therefore, despite the fact that myofibres were isolated from the soleus muscle and had presumably heterogeneous fibre-type profiles, the toxic effects of notexin did not appear to be differential as has been reported previously (reviewed, Harris 2003). The mean numbers of cells surrounding myofibres exposed to the different concentrations of notexin are shown in **Fig. 5.15**. At the highest concentrations (10 µg/ml and 1 µg/ml), cells took on a granular appearance, and some appeared to die and detach from the

substrate. The numbers of desmin⁺ cells surrounding single myofibres after 120 h in culture was compared using the Kruskal-Wallis test. The differences between the six treatment groups were found to be extremely significant ($p < 0.0001$). Dunn's multiple comparisons test was used to compare the control individually with each of the five different treatment groups. The difference between the control and notexin 10 $\mu\text{g/ml}$ was found to be very significant ($p < 0.001$). The difference between the control and notexin 1 $\mu\text{g/ml}$ was found to be significant ($p < 0.05$). Differences between the control and the other three treatment groups were not significant ($p > 0.05$). These data show that *in vitro*, notexin does not stimulate proliferation but is toxic to proliferating satellite cell-derived myoblasts in a dose-dependent manner.

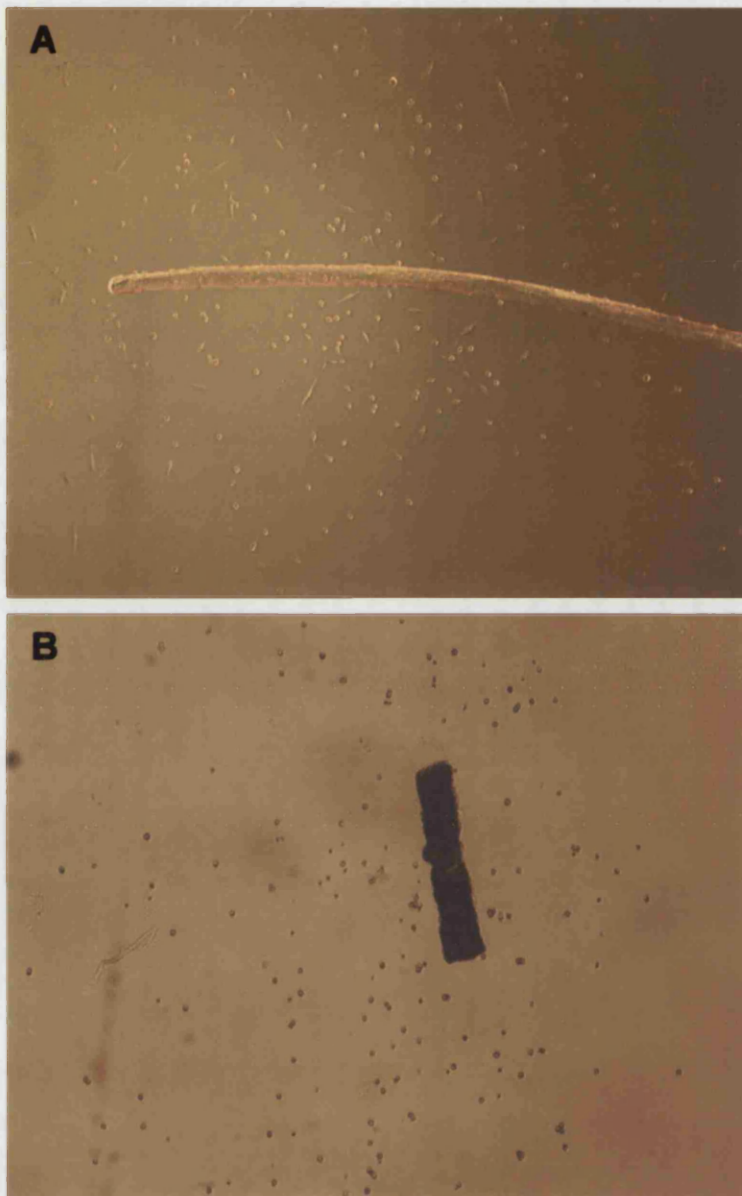


Fig. 5.14. Effect of notexin on soleus single myofibre cultures. In a control culture (A) the adherent myofibre is intact and is surrounded by proliferating satellite cell-derived myoblasts. In a culture exposed to notexin (10 µg/ml) 24 h previously (B) the myofibre has hypercontracted, and many of the surrounding myoblasts appear sick, and subsequently detached from the substrate. Image capture of live cells. Original magnification x50.

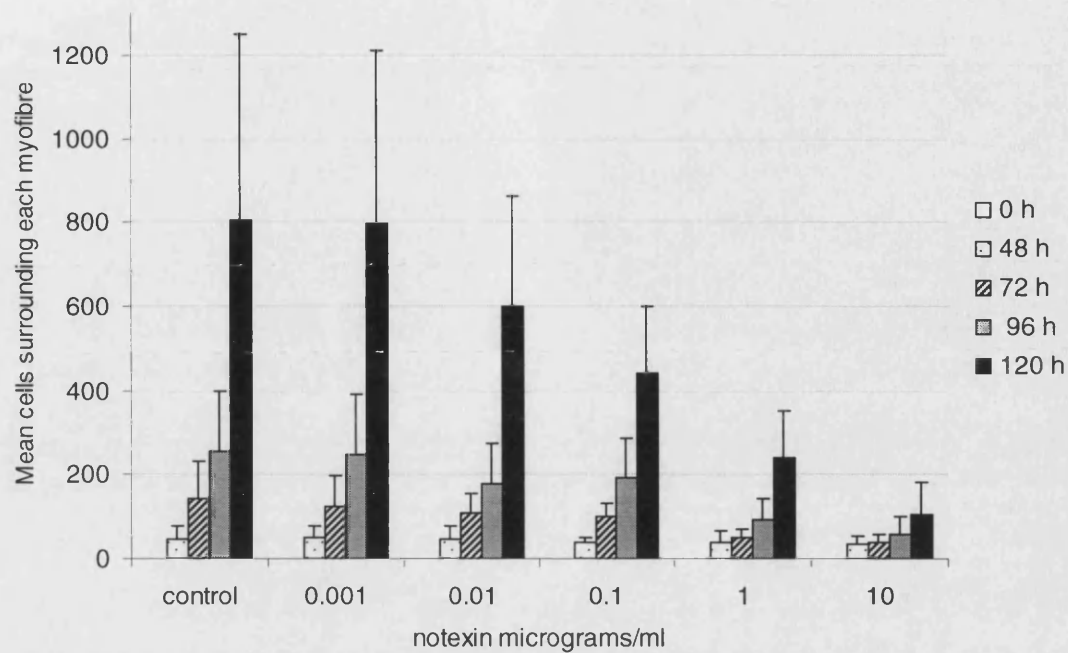


Fig. 5.15. Bar graph showing the effect of notexin on satellite-derived cell proliferation in soleus single myofibre cultures. After 48 h in culture, cells were exposed overnight to various concentrations of notexin, and then gently washed and returned to normal culture conditions for a further 72 h. In other *in vivo* experiments notexin was injected at 10 $\mu\text{g/ml}$. Here *in vitro*, at notexin concentrations from 0.01-10 $\mu\text{g/ml}$ all myofibres hypercontracted, excepting 1 single myofibre that survived exposure to 0.01 $\mu\text{g/ml}$. Control myofibre cultures and those exposed to 0.001 $\mu\text{g/ml}$ notexin all survived for the duration of the experiment (**Fig. 5.14**). Error bars represent SD.

5.4 Investigation of the effect of myofibre damage on myoblast proliferation

5.41 Aim

Exposure of single myofibre cultures to notexin was found to have toxic effects on both the parent myofibre and its associated satellite cell-derived myoblasts, and did not enhance myoblast proliferation (5.3). *In vivo*, satellite cell proliferation is stimulated by muscle tissue damage (reviewed, Bischoff 1994); however, it is not known whether this effect is significantly mediated by direct signalling from damaged myofibres, or via signalling from other (e.g. inflammatory) phenotypes present within the regenerative milieu. The objective of this study was to use single myofibre cultures to investigate whether the proliferation of satellite cell-derived myoblasts was altered after the parent myofibre had been subjected to physical damage.

5.42 Effect of myofibre damage on the proliferation of satellite cell-derived myoblasts in single myofibre cultures

Single myofibres were isolated from the EDL and soleus muscles of one 28-day-old C57 Bl/10 mouse and from the EDL muscles of a second 42-day-old C57 Bl/10 mouse and cultured individually in the wells of 24-well-plates (2.21). After 48 h, examination of cultures showed that satellite cells had migrated away from parent myofibres and started to proliferate. At this stage, 50% of the medium in cultures was removed and replaced with fresh warmed plating medium, added gently down the side of each well to avoid disturbing myofibre. In some cultures (24/48 soleus myofibres, 45/89 total EDL myofibres), focal damage was caused to adherent myofibres by crushing a central segment with a heat-polished Pasteur pipette, taking care not to touch surrounding mononucleate cells. This treatment resulted in part or all of the myofibre hypercontracting, and in some cases, detaching from the substrate. Remaining cultures served as undamaged controls. Cultures were maintained for a further 72 h post-damage and then fixed and immunostained for desmin protein to confirm the myogenic lineage of emanating cells (Bockhold *et al.* 1998) (2.74) (Fig. 5.16). The numbers of desmin⁺ and desmin⁻ cells surrounding each myofibre were counted (Figs. 5.17 & 5.18). 2/23 (8.7%) of control soleus myofibre cultures respectively had 1.1% and 1.5% desmin⁻ cells. 1/24 (4.2%) of damaged soleus

myofibre cultures had 1.7% desmin⁻ cells. 1/44 (2.3%) of control EDL myofibre cultures had 19.6% desmin cells. 1/45 (2.2%) of damaged EDL myofibre cultures had 15.3% desmin⁻ cells. In all other cultures, all cells surrounding parent myofibres expressed desmin protein.

The Kolgomorov-Smirnov test (see Appendix) was used to compare the distribution of counts of desmin⁺ cells emanating from control and damaged myofibres. There was no statistically significant difference between the numbers of desmin⁺ cells surrounding intact or damaged EDL or soleus parent myofibres ($p \geq 0.05$). These data suggest that *in vitro*, damaged myofibres do not release substances that either augment or retard the proliferation of satellite cell-derived myoblasts.

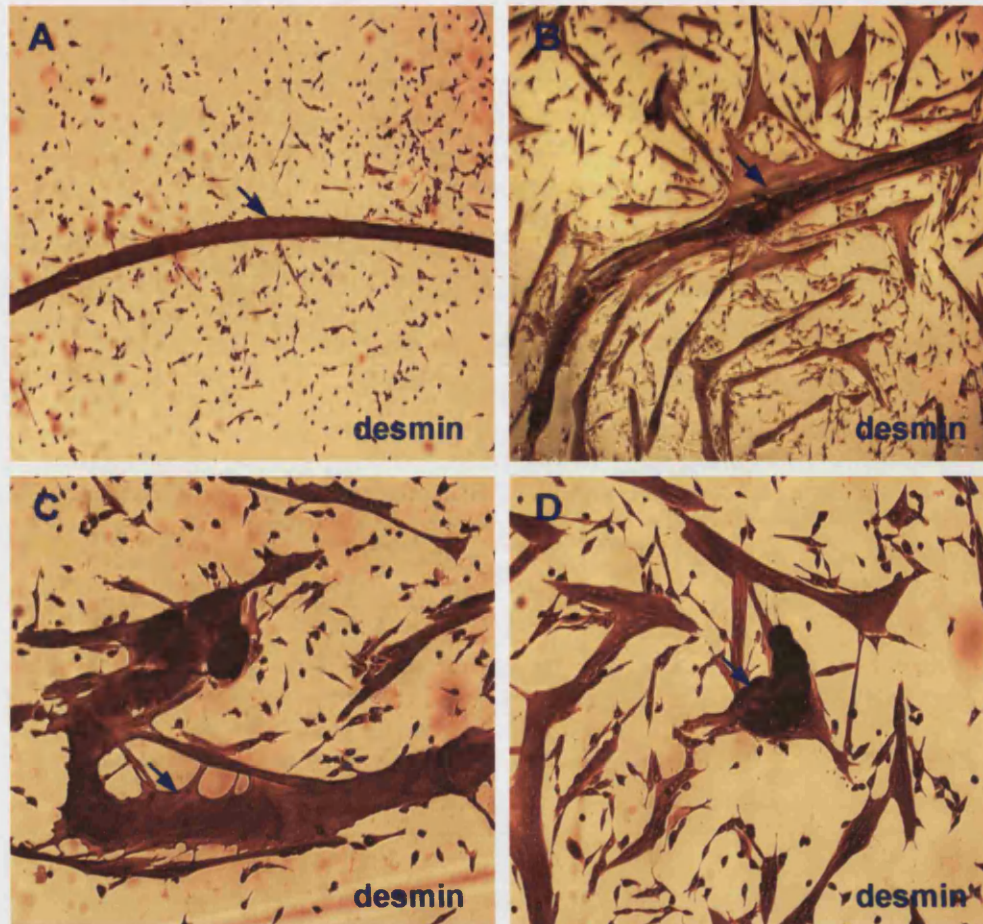


Fig. 5.16. Effect of myofibre damage in single myofibre cultures. Single myofibres (arrows) isolated from the EDL (A, C) or soleus (B, D) muscles of wild-type mice were maintained as adherent cultures for 120 h, and immunostained for expression of desmin protein (peroxidase detection protocol). Control cultures (A, B) and cultures in which the parent myofibre was physically damaged after 48 h in culture (C, D), 72 h before fixation and staining. There was no statistically significant difference between the numbers of desmin+ cells surrounding control and myofibre-damaged cultures (Kolgomorov-Smirnov $p \geq 0.05$). Original magnification x50 (A-B) or x100 (C-D).

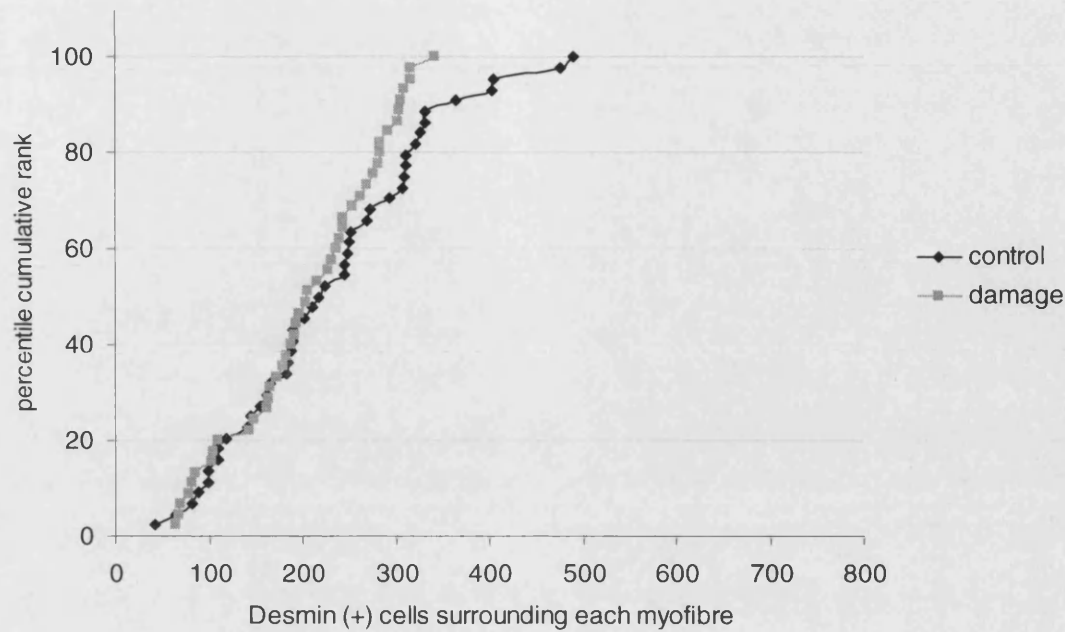


Fig. 5.17. Cumulative plot of data from individual cultured EDL myofibres, ranked according to the number of desmin⁺ cells surrounding each explant. To normalise the data for sample size, the individual ranks are expressed as a percentage of the total rank for each group. After 48 h in culture, adherent myofibres in the 'damage' group were focally damaged by crushing a central segment with a heat-polished Pasteur pipette, without disturbing cells that had previously emanated from the explant. This caused part or all of the myofibre to hypercontract, and in some cases to detach from the substrate (5.16). Cultures were maintained for a further 72 h before fixation and desmin immunocytochemistry. Pooled data from two experiments.

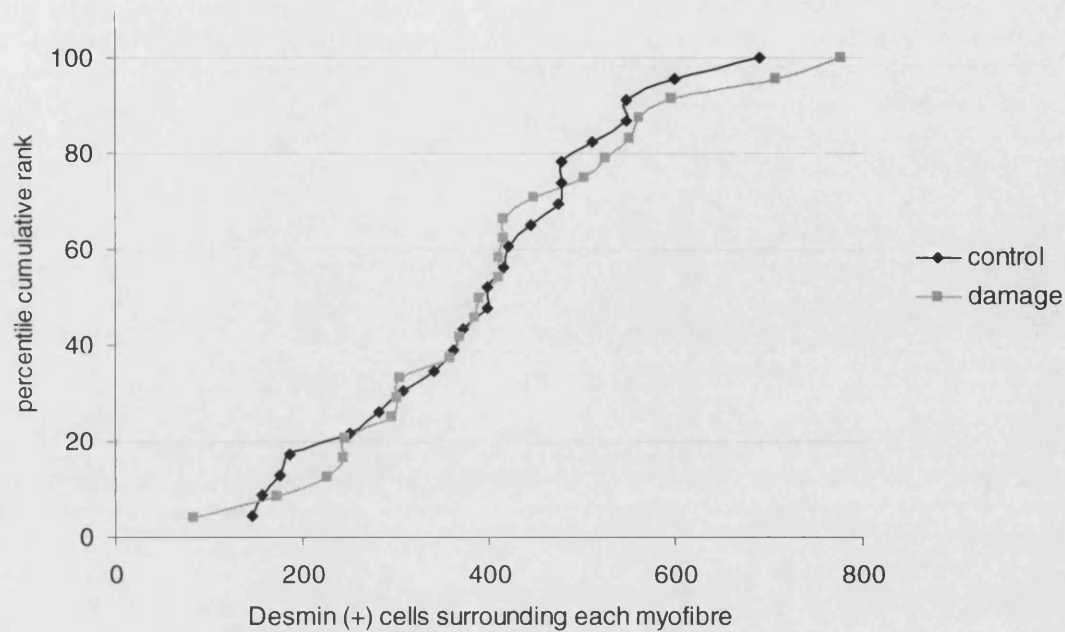


Fig. 5.18. Cumulative plot of data from individual cultured soleus myofibres, ranked according to the number of desmin⁺ cells surrounding each explant. To normalise the data for sample size, the individual ranks are expressed as a percentage of the total rank for each group. After 48 h in culture, adherent myofibres in the 'damage' group were focally damaged by crushing a central segment with a heat-polished Pasteur pipette, without disturbing cells that had previously emanated from the explant. This caused part or all of the myofibre to hypercontract, and in some cases to detach from the substrate (**Fig. 5.16**). Myofibres were maintained in culture for a further 72 h before fixation and desmin immunocytochemistry.

5.5 The myogenic potential of satellite cells in non-irradiated graft sites

5.51 Aim

Previous unpublished attempts to graft EDL single myofibres into non-irradiated host sites failed to generate donor-derived muscle formation. However, engrafted muscles were analysed at only the three week timepoint, and were not challenged with damage (L. Heslop, PhD Thesis 2001). Hypothetically, the lack of muscle formation could have been due to either death of the grafted cells, or non-proliferation of surviving cells in a sub-optimal environment. Another previous study showed that grafted C2C12 cells survived in non-irradiated environments but formed less muscle than in irradiated environments (Morgan *et al.* 2002). The objective of the present experiment was to determine whether satellite cells persist in non-irradiated graft sites and can be stimulated to enter an active myogenic program after severe damage, or whether the the myogenic potential of grafted satellite cells is entirely absent in non-irradiated environments.

5.52 Grafts of single myofibres into non-irradiated host sites

Single myofibres were isolated from The EDL muscles of a 43-day-old *Myf5^{nLacZ/+}* mouse and prepared for grafting as previously described (2.21, 3.42). The donor mouse used was the same as that used in 5.25. Single myofibre grafts were inserted into the non-irradiated TA muscles (n=20) of ten *mdx*-nude host mice (2.55, 3.42). Four weeks post-grafting, mice were anaesthetised and the right engrafted TA muscle of each animal damaged by injection of notexin (2.56). The contralateral engrafted TA muscles served as non-damaged controls. Muscles were removed five weeks after grafting and one week after notexin damage, and processed for immunohistochemistry (2.61). Four serial sections were collected at 100 µm intervals throughout entire muscle bodies (2.62). The first set of sections was stained in X-gal solution (2.77). β-gal activity could not be identified in any sections of any engrafted muscles, either those with, or without notexin damage (Fig. 5.19, Table 5.4).

Origin of graft	No. of grafts	Pre-irradiation of graft site	Notexin damage to engrafted muscle	Engrafted muscles with ≥ 1 β -gal ⁺ nucleus
<i>Myf5</i> ^{nLacZ/+} EDL	10 (right host TA muscles)	no	yes	0/10
<i>Myf5</i> ^{nLacZ/+} EDL	10 (left host TA muscles)	no	no	0/10

Table 5.4. Absence of muscle formation by grafts of *Myf5*^{nLacZ/+} EDL single myofibres inserted into non-irradiated host TA muscles. Muscles were removed 5 weeks after grafting. Notexin was injected into the right engrafted muscle of each mouse 1 week before removal. The contralateral left engrafted muscle served as the non-damaged control.

This result shows that single myofibre grafts do not generate any MPC capable of forming muscle in non-irradiated sites. The data could represent a failure of cell survival, or alternatively an absence of myogenic potential in the presence of the normal complement of host satellite cells.

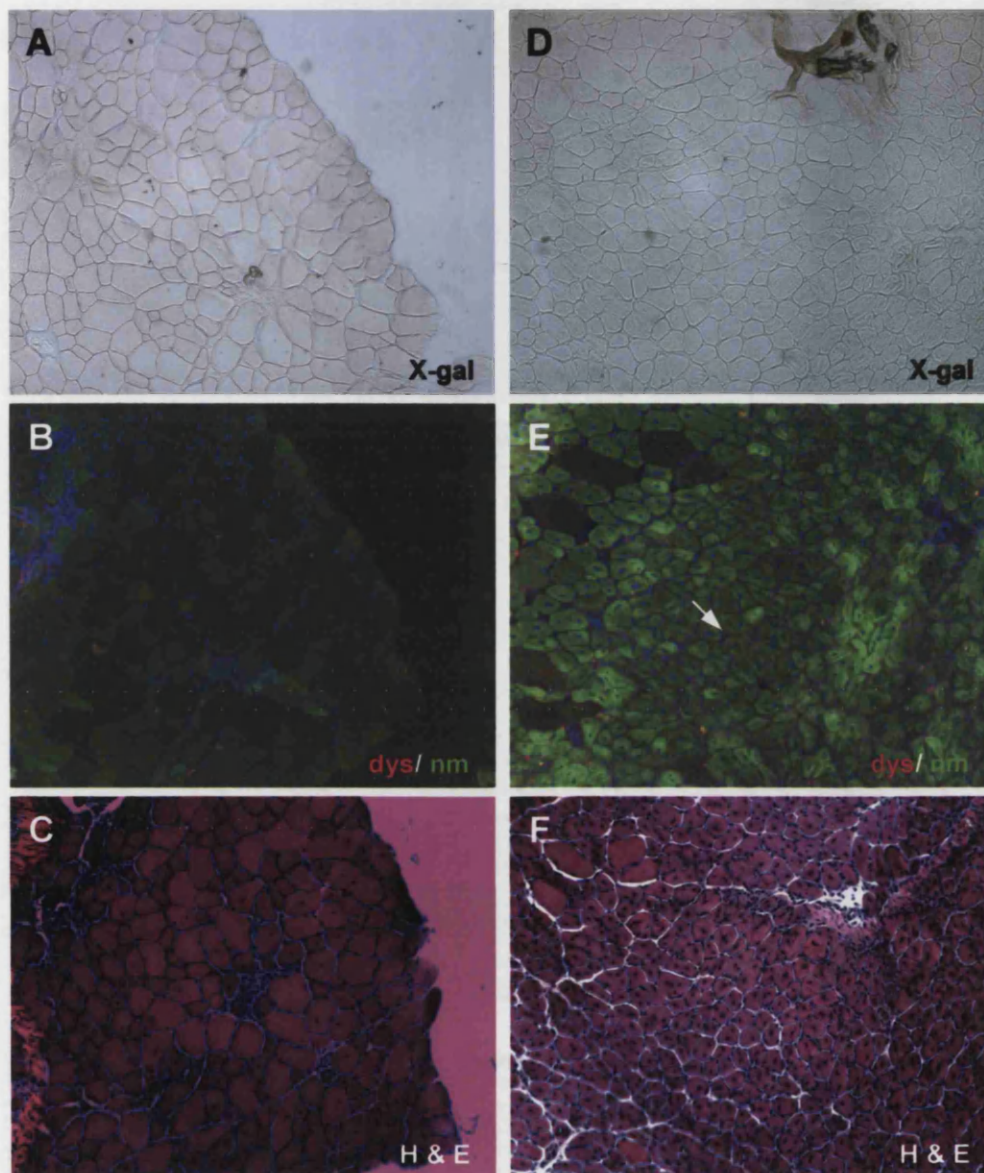


Fig. 5.19. Absence of donor-derived muscle formation in non-irradiated *mdx*-nude TA muscles engrafted with a single *Myf5*^{nLacZ/+} EDL myofibre, either with (D-F) or without (A-C) subsequent notexin-induced damage. Serial sections: stained in X-gal (A, D), immunohistochemistry for dystrophin and neonatal myosin (B, E), and stained with H & E (C, F). 1 week after notexin injury (D-F), non-irradiated muscles contain numerous small, centrally nucleated myofibres (F) that express neonatal myosin (E). However, the lack of expression of β -gal (A, D) and dystrophin (B, E) in both undamaged (A-C) and damaged (D-F) engrafted muscles shows that there is no donor contribution. A single weakly dystrophin⁺, presumably revertant, myofibre is arrowed (E). Original magnification x100.

5.6 Comments on results

The data derived from experiments in this chapter demonstrate that all soleus and TA single myofibre grafts (5.23, 5.24) and the majority of EDL myofibre grafts (5.22) generate at least one cell capable of undergoing rapid clonal expansion in response to severe damage. In all cases, donor-derived regenerate muscle formation was localised to either a single compact cluster, or less frequently, two or three discrete foci. These clusters could either have been of clonal origin, or have originated from several closely-associated progenitors. Since notexin caused severe damage to more than 75% of the area of injected muscles, it is unlikely that clusters were generated from more widely-distributed precursors that migrated towards a single focus.

In muscles engrafted with EDL (5.22) or TA (5.24) myofibres the frequency of muscle formation was increased in notexin-damaged muscles compared to non-damaged controls (Table 5.1). In soleus myofibre grafts (5.23), whilst the grafting efficiency was 100% in both notexin-damaged and non-damaged engrafted muscles, the quantity of muscle formed 1 week after notexin injection was greater than the quantity of muscle formed in the absence of notexin damage. Soleus myofibres have more satellite cells than either EDL or TA myofibres (3.41) and in these experiments, their regenerative potential was found to be greater than myofibres isolated from either of the other muscles. Taken together, these data suggest that notexin damage recruited previously inactive (possibly quiescent) precursors into an active myogenic program, instead of, or as well as, upregulating the proliferative activity of already-active precursors. However, the data do not distinguish whether the notexin-activated cells were the originally-grafted satellite cells which had hitherto remained persistently quiescent, or newly-formed satellite cells generated during a previous myogenic event, or other donor-derived progeny located outside the satellite cell compartment.

Without the stimulus of notexin-damage, both the frequency and overall efficiency (Table 5.1) of muscle formation was greater at the five week timepoint than that observed in previous experiments analysed at the three week timepoint (3.4). This implies that satellite cell recruitment into active myogenesis also occurs in the absence of additional experimental pressure, either in response to local bouts of

degeneration and regeneration which are characteristic of the host *mdx* muscles, or spontaneously as a result of pre-programmed cell cycle re-entry determined by cell-intrinsic factors. Most strikingly, the efficiency of muscle formation by TA myofibre grafts at the five week timepoint greatly exceeded that at the 3 week timepoint, both in absolute terms and relative to muscle formation by EDL and soleus myofibres. These data suggest that TA satellite cells have similar myogenic potential to EDL and soleus satellite cells, but that their progeny proliferate (or possibly differentiate) at a slower rate.

The finding that myogenic cells derived from a proportion of EDL myofibre grafts are capable of responding to at least two bouts of severe damage (2.25) demonstrates that satellite cells can maintain their own population in terms of retaining functional potential in addition to generating new anatomically- and phenotypically-defined satellite cells, as was shown in Chapter 4. Since in the majority of cases each graft presumptively contained several satellite cells, it is possible, albeit improbable, that after the second bout of damage muscle regeneration was derived from a cell which had resisted activation during the first bout.

Though notexin-induced damage elicits a robust regenerative response from satellite cells *in vivo* (5.2), in the tissue culture environment exposure to notexin retards the proliferation of satellite cell-derived myoblasts (5.3). This disparity may reflect differences in the concentrations to which satellite cells were exposed to. *In vivo*, the injected concentration (10 µg/ml) is likely to be considerably higher than the concentration to which the majority of satellite cells are exposed after dispersal of the injected fluid throughout the muscle body. At lower concentrations, the *in vitro* toxicity of notexin was much less marked (Fig. 5.15), probably a closer representation of its actual effects *in vivo*. Alternatively, the *in vivo/in vitro* disparity may be attributable to factors related directly to the tissue culture environment itself. Oxidative myofibres are reportedly more sensitive to myotoxic agents than glycolytic myofibres (1.6). Cells are likely to be subject to greater oxidative stress in a tissue culture environment with 21% O₂ than *in vivo* with 6% O₂, a possible consequence of which could be greater sensitivity to myotoxins such as notexin. In addition, it is possible that quiescent satellite cells are less sensitive to notexin than the actively-proliferating satellite cell-derived myoblasts examined in this study. It would be

difficult to test the effects of notexin on anatomically-defined quiescent satellite cells *in vitro*, firstly because satellite cells rapidly activate on exposure to the tissue culture environment (Zammit *et al.* 2002), and secondly because notexin exposure causes myofibres to hypercontract, preventing them from adhering in culture and making them too optically dense for fluorescence immunocytochemistry. Consequently, it is difficult to determine whether the toxic effects of notexin on proliferating myoblasts *in vitro* are really representative of its effects on quiescent satellite cells *in vivo*. Notwithstanding, the data strongly suggest that satellite cell proliferation is stimulated by the tissue damage caused by notexin, rather than by a direct effect of notexin itself.

Grafts of single myofibres into non-irradiated sites failed to generate a single donor-derived nucleus that could be identified as such by expression of Myf5- β -gal fusion protein, either after or without notexin-induced damage (**Table 5.4, Fig. 5.19**). Although it might be predicted that the presence of proliferation-competent host satellite cells would reduce the graft-derived regeneration through direct competition, it is notable that the donor contribution was completely absent. In contrast to this finding, both primary neonatal myoblasts (Partridge *et al.* 1989) and immortalised C2C12 myoblasts (Morgan *et al.* 2002) form muscle in non-irradiated environments. This difference suggests that the mechanism of muscle formation by satellite cells in single myofibre grafts may differ from the mechanism of muscle formation by grafted myoblasts which are outside the basal lamina.

In summary, the results of the experiments described in this chapter demonstrate that satellite cells respond to damage by undergoing rapid and sustained proliferation to both regenerate large areas of new muscle and maintain a reserve population capable of participating in subsequent regenerative events. The behaviour of satellite cells in single myofibre grafts recapitulates the endogenous process of regeneration in whole muscles and suggests that satellite cells constitute the basal origin of adult muscle regeneration.

Chapter 6

General Discussion

This thesis has aimed to analyse the stem cell potential of satellite cells by formally investigating their contribution to skeletal muscle regeneration. In this chapter, results are consolidated, placed in the context of current knowledge of muscle regeneration and satellite cell biology, and their contribution to the field discussed.

6.1 Stem cell activity of satellite cells in myofibre grafts

Skeletal muscle has a remarkable ability to regenerate functional tissue within a few days of severe injury (Studitsky 1964, reviewed, Carlson 1986). Though the ability of satellite cells to contribute to adult myogenesis is beyond doubt both *in vivo* (Moss & Leblond 1971, Snow 1978) and *in vitro* (Rosenblatt *et al.* 1995), the rapidity with which regeneration proceeds (reviewed, Carlson 1986), has prompted some workers to predict that this relatively minor population is not the only significant source of myonuclei in regenerating muscles (Reznik 1970, Seale *et al.* 2001, LaBarge & Blau 2002). Others have disputed this, demonstrating that *in vitro*, the satellite cells associated with each single myofibre have sufficient proliferative potential to completely replace the number of nuclei in the parent myofibre within four days (Zammit *et al.* 2002).

Following muscle injury, inflammatory cells rapidly infiltrate the regenerative milieu (reviewed, Partridge 1982). Whilst it has been demonstrated that cells derived from the circulatory system can contribute at low frequency to myofibres (Ferrari *et al.* 1998, Camargo *et al.* 2003, Torrente *et al.* 2004), the severe growth deficit generated by local irradiation of juvenile muscles (Wakeford *et al.* 1991, Quinlan *et al.* 1995) implies that the most significantly myogenic cells are both located within the tissue, and sensitive to radiation. It has been shown that cell types which are phenotypically distinct from satellite cells, but believed to be tissue-resident, can be recruited into a myogenic program in response to injury (Polesskaya *et al.* 2003). However, *in vivo*,

these non-satellite cell phenotypes have not yet been unequivocally linked with significant myogenic activity.

This thesis has demonstrated that *in vivo*, the satellite cells associated with a single grafted myofibre have the potential to not only regenerate the nuclei of their parent myofibre (Zammit *et al.* 2002), but to make a significant contribution to the nuclei of more than a hundred other myofibres. This is the first time that such substantial myogenic activity has been formally localised to a defined phenotype. The data predict that satellite cells have sufficient proliferative, myogenic, and self-renewal potential to form the major, or possibly only, cellular source in the rapid and robust regenerative process previously described by other workers (Studitsky 1964, Carlson 1968, 1970, Luz *et al.* 2002). Although most quiescent satellite cells express certain muscle-specific proteins such as Myf5 (Beauchamp *et al.* 2000), M-cadherin (Irintchev *et al.* 1994, Kuschel *et al.* 1999) and probably desmin (personal communication, P. Zammit), this thesis demonstrates that they are not merely committed precursors held in a temporary differentiation block, but have the endogenous potential to function as tissue-specific stem cells.

6.2 Evidence for a myofibre-dependent activation event

In Chapter 3, it has been shown that in similar host environments, single myofibres form muscle with greatly increased efficiency compared to primary myoblasts derived from equivalent muscles, despite the fact that many (or perhaps most) primary myoblasts are likely to have been derived from the satellite cell position. These data demonstrate the existence of a phenotype which does not retain its full myogenic potential in conventional preparations of myoblasts. The mitotic quiescence of satellite cells may give them a survival advantage over proliferating cells. Single myofibres may represent highly-purified populations of the most potent myogenic precursors which in heterogeneous populations are greatly diluted. Alternatively, the data may describe a phenotype whose myogenic potential is non-autonomous, but is dependent on a component of the satellite cell niche.

The number of cells that survive in grafts of immortalised or primary myoblasts is only about 2% of the total population (Beauchamp *et al.* 1999). In this thesis, this

figure equates to approximately 10^4 cells surviving in grafts of 5×10^5 immortalised myoblasts and 10^3 desmin⁺ cells surviving in grafts of 5×10^4 primary myoblasts. Even in the largest possible outliers, the maximum number of satellite cells surviving in single myofibre grafts was necessarily always less than 50, and in EDL single myofibre grafts usually less than 10. Whilst the 10 cells that would be predicted to survive in grafts of 500 immortalised myoblasts formed a maximum of 12 new myofibres, the less than 10 satellite cells associated with an EDL myofibre formed a maximum of 189 myofibres at the 3 week timepoint. Thus the increased myogenicity of myofibre grafts cannot be wholly attributed to purification of a myogenic stem cell which forms a small subset of other myoblast preparations. It has been shown that the 2% of myoblasts which survive grafting are those which, in tissue culture, are slow-cycling and do not incorporate ³H-thymidine in the immediate period before transplantation (Beauchamp *et al.* 1999). Hypothetically, the mitotic quiescence of adult satellite cells (Schultz *et al.* 1978) might enhance their survival in myofibre grafts, but equally, primary myoblasts which have never been maintained in tissue culture might be expected to have a survival advantage for the same reason.

Purity of the myogenic population and potentially enhanced frequency of survival cannot fully account for the very high efficiency of muscle formation by grafted single myofibres. It is therefore strongly implied that the satellite cell niche has a role in maintaining stem cell potential. The stem cell potential of many phenotypes, including intestinal epithelial stem cells (Kaeffer 2002), skin epithelial stem cells (Blanpain *et al.* 2004) and neural stem cells (Song *et al.* 2002b, Shen *et al.* 2004), is critically controlled by a component of their endogenous niche, and therefore non-autonomous. The high frequency of single HSC engraftment demonstrates that these stem cells have an excellent ability to home to the appropriate niche (Matsuzaki *et al.* 2004).

Whilst the association of satellite cells with their parent myofibre appears to be an important factor determining myogenic potential, the large amounts of muscle formed by single myofibre grafts are evidence that satellite cell progeny do not remain associated with their parent myofibre for an extended period. Experiments described in Chapter 3 demonstrated that though enzymatically-disaggregated myoblasts formed muscle relatively inefficiently, grafts of single myofibres generated muscle very

efficiently, even when the graft was inserted into a notexin-treated site in which the myofibre could not have remained viable for long. In addition, *in vitro* experiments described in Chapter 5 showed that the proliferation of satellite cell-derived myoblasts was not increased when damage was caused to the parent myofibre. These data predict a mechanism whereby an initial myofibre-associated activation event is dependent on a component of the satellite cell niche, but the subsequent proliferation of satellite cell progeny is myofibre-independent.

Though terminally differentiated, myonuclei retain the ability to alter their expression profiles in response to changing workloads or after damage. Myofibres can switch expression of MyHC isoforms in response to changes in the electrical activity of their attendant nerve (reviewed, Hughes & Salinas 1999, Pette & Staron 2000). The myonuclei of isolated EDL and TA myofibres re-activate the *Myf5* locus in response to denervation and serum stimulation, though the myonuclei of soleus myofibres do not (Zammit *et al.* 2002). Since myonuclei are postmitotic, upregulation of myonuclear *Myf5* expression (Zammit *et al.* 2002) does not precede mitotic activity as it does in satellite cells (Cornelison & Wold 1997), but speculatively may be indicative of some form of signalling mechanism between myofibre and satellite cell. This thesis did not attempt to determine the period for which grafted myofibres remained viable after insertion into the host muscle. It would be difficult to do so, since the extremely delicate nature of single myofibres means that labelling with any form of dye could itself have a negative effect on viability. It is probable that outside the tissue culture environment, and in the absence of nerves and vasculature, myofibres do not persist for long. However, since *in vitro*, most satellite cells become activated at the protein level within six hours of isolation (Zammit *et al.* 2002), a hypothetical myonuclear signalling event would not necessarily require persistence of grafted myofibres for long.

Minced muscle grafts regenerate functional tissue with remarkable efficiency (reviewed, Carlson 1986), despite the fact that the original myonuclei rapidly become pyknotic (Snow 1977a, 1977b), and cannot be supposed to have a persistent signalling role. Satellite cells remain viable beneath the relatively intact basal laminae (Snow 1977a, 1977b). The basal lamina also survives after other forms of severe damage, such as barium chloride treatment, and acts as a scaffold for subsequent regeneration

(Caldwell *et al.* 1990). It could, therefore, be involved in determining satellite cell fate. Proliferation and quiescence of arterial smooth muscle cells have been shown to be controlled by integrin signalling, mediated by extracellular matrix proteins (Hedin *et al.* 1999), and proliferation and quiescence of skin stem cells are similarly thought to be mediated through basal lamina signalling (Blanpain *et al.* 2004). This suggests the possibility that the basal lamina of myofibres could play an equivalent role in mediating the activation status of satellite cells.

The increase in frequency of muscle formation over time and in response to severe damage, shown in Chapter 5, demonstrates that some grafted satellite cells remained inactive in engrafted muscles, and retained latent stem cell potential after the original parent myofibre had, in all probability, been degraded. In the irradiated graft site, donor satellite cells could have been recruited or homed to the satellite cell-depleted niches of host myofibres, and thereby acquired a position in which they were amenable to appropriate signalling mechanisms. It has been proposed that the ability to home to a niche appropriate for their survival and proliferation should be one of the qualities by which stem cells are defined (Matsuzaki *et al.* 2004). The high frequency of satellite cell engraftment demonstrated by this thesis suggests that satellite cells satisfy this criterion, albeit, similarly to HSC (Smith *et al.* 1991, Osawa *et al.* 1996, Matsuzaki *et al.* 2004), in irradiated graft sites. It has been shown in Chapter 5 that single myofibre grafts do not generate muscle or new satellite cells in non-irradiated graft sites. Isolated myoblasts, however, have previously been shown to form muscle in non-irradiated graft sites (Partridge *et al.* 1989, Morgan *et al.* 2002), though with lower efficiency than in irradiated sites (Morgan *et al.* 2002). This difference was found to be attributable to reduced proliferation rather than reduced survival (Beauchamp *et al.* 1999, Morgan *et al.* 2002). Hodgetts & Grounds (2003) showed that in the immunocompetent host, the improved efficiency of muscle formation in irradiated sites is linked to a reduction in numbers of host immune cells. However, the experiments described in this thesis (and also those of Morgan *et al.*) were carried out in *mdx*-nude hosts, which lack thymi and are highly immunotolerant of mouse allografts (personal communication, T. Partridge). It is therefore likely that the observed differences between the behaviour of grafted satellite cells in irradiated and non-irradiated environments resulted from a factor independent of host immune response. Speculatively, the absence of muscle formation in non-irradiated sites could

be due to the inability of donor satellite cells to home to a suitable niche in the presence of the intact population of host satellite cells. The sustained absence of muscle formation five weeks after grafting, and moreover after severe myotoxin-induced damage, implies that satellite cells do not remain viable (or at least lose their functional attributes) if they are prevented from homing to this niche within a certain period of grafting. This hypothesis could be tested further using grafts into host muscles that are depleted of satellite cells for reasons other than exposure to irradiation, for instance into the muscles of adult *Pax7^{-/-}* animals (Seale *et al.* 2000, Oustanina *et al.* 2004).

6.3 Self-renewal of the adult satellite cell compartment

It was Moss and Leblond (1971) who originally proposed that the adult satellite cell compartment could be maintained by self-renewal. More recent studies have shown that satellite cells can be derived from transplanted populations of myoblasts (Blaveri *et al.* 1999, Heslop *et al.* 2001, Cousins *et al.* 2004) and that *in vitro*, isolated myoblasts (Baroffio *et al.* 1996, Yoshida *et al.* 1998) and myofibre-adherent satellite cells (Zammit *et al.* 2004a) generate reserve populations that remain proliferation-competent when the majority population commits to differentiation. In experiments described in Chapter 4, this thesis has demonstrated that defined satellite cells can generate new defined satellite cells during the course of a regenerative event, and that the satellite cell compartment is therefore capable of renewing its own population. The satellite cells associated with a single grafted myofibre were found to contribute new satellite cells to the populations associated with numerous other myofibres, thereby both expanding the original number of satellite cells as well as contributing differentiated myonuclei. The presence of satellite cells on very immature, centrally nucleated myofibres implies that satellite cells and myonuclei were generated within a similar timescale, rather than by temporally separable events. This ability to activate and generate proliferation-competent progeny of which a subset return to an inactive or quiescent state has been recently demonstrated using single myofibres in suspension culture (Zammit *et al.* 2004a). However, satellite cells were found to have a very limited proliferative capacity, undergoing only a few divisions before the majority population committed to differentiation (Zammit *et al.* 2004a). This thesis has demonstrated that *in vivo*, satellite cell progeny can undergo very extensive

proliferation to generate large numbers of myofibres and new satellite cells. Hypothetically, this process could involve sequential small discrete regenerative events, whereby an activated cell both generates a small number of differentiation-committed progeny and replaces itself with a proliferation-competent cell that subsequently derives the next cohort after its sibling population have differentiated. Alternatively, all progeny may exit the cell cycle relatively synchronously after a single prolonged episode of proliferation. Evidence derived from previous studies (Barroffio *et al.* 1996, Zammit *et al.* 2004a) suggests that the former possibility is most probable.

Stem cells are thought to self-renew by the asymmetric division of a founder cell to generate two daughter cells, of which only one gives rise to differentiation-committed progeny (reviewed, Hall & Watt 1989, Potten & Loeffler 1990, Morrison *et al.* 1997, Weissman *et al.* 2000). However, there is evidence that under some circumstances, stem cells can be renewed via alternative mechanisms. Intestinal crypts contain both self-renewing stem cells, and their progeny, transit amplifying cells, which are programmed to terminally differentiate after a few rounds of division, and do not normally self-renew. However, if the stem cell population is ablated, transit amplifying cells can revert to a stem cell phenotype and regenerate the entire crypt, including the primitive stem cell population (reviewed, Marshman *et al.* 2002, Potten 2004). Most quiescent satellite cells do not contain MyoD protein (Zammit *et al.* 2002) or *MyoD* mRNA (Cornelison & Wold 1997), and though Myf5 protein is present (Beauchamp *et al.* 2000), *Myf5* mRNA is not detectable (Cornelison & Wold 1997). Satellite cell activation is characterised by the almost ubiquitous upregulation of *Myf5* and *MyoD* mRNA and protein expression (Cornelison & Wold 1997, Cooper *et al.* 1999, Zammit *et al.* 2002, 2004a), which is retained by the proliferative progeny (Zammit *et al.* 2002). This thesis has demonstrated that the progeny of satellite cells can undergo extensive *in vivo* or *in vitro* proliferation before returning to the satellite cell niche. This implies the existence of a renewal mechanism analogous to the stem cell-reversion of intestinal crypt transit amplifying cells (reviewed, Marshman *et al.* 2002, Potten 2004), whereby satellite cell progeny enter an active myogenic program from which a subset subsequently withdraw, maintaining the precursor population by reverting to a quiescent state. Perhaps similarly, HSC have been shown to exist in a state of reversible mitotic quiescence whereby at least 75% of the population is

quiescent at any one timepoint, but almost the entire population is recruited into the cell cycle during a thirty day period (Bradford *et al.* 1997, Cheshier *et al.* 1999). However, there is no evidence that the progeny of HSC can express markers of commitment to a particular myeloid lineage and subsequently return to mitotic quiescence, a respect in which they may differ from the progeny of satellite cells.

Previously, it has been demonstrated that satellite cells associated with single myofibres in suspension culture generate reserve progeny with a mechanism suggestive of self-renewal (Zammit *et al.* 2004a). In Chapter 4 it was shown that the satellite cells associated with one grafted myofibre can generate progeny which form new satellite cells on many other myofibres, demonstrating that satellite cells can be formed outside of the original parental niche. In addition, a Pax7⁺ satellite cell-like phenotype was generated as a reserve population in differentiated single myofibre cultures (Chapter 4), from cells unequivocally outside the satellite cell niche. Therefore although it is possible, and perhaps highly probable, that the satellite cell niche has a role in maintaining the undifferentiated and quiescent state of satellite cells, these findings predict that cells committed to becoming satellite cells are originally generated outside of the niche into which they are subsequently recruited.

Skeletal muscles maintain the ability to regenerate throughout several bouts of damage (Luz *et al.* 2002). This thesis has shown that the satellite cells associated with a single grafted myofibre generate progeny which can both participate in regeneration induced by the chronic *mdx* myopathy, and in at least two acute regenerative episodes induced by application of myotoxin. This demonstrates that satellite cells can maintain a functional myogenic population not only throughout the relatively moderate demands of postnatal growth and focal myopathic damage, but whilst undergoing rapid clonal expansion within the disorganised regenerative milieu of notexin-damaged muscle. These data predict that the satellite cell compartment constitutes a self-sufficient regenerative source, and is not dependent on a contribution from other cells either derived from other niches within the tissue, or from the circulation. Though anatomically-defined satellite cells which express appropriate phenotypic markers can be derived from grafts of bone marrow cells (LaBarge & Blau 2002, Dreyfus *et al.* 2004), data linking these cells to any significant myogenic activity is lacking. Furthermore, previous studies have shown that local

exposure of mouse muscles to 18 Gy irradiation results in a long-term growth deficit (Wakeford *et al.* 1991, Quinlan *et al.* 1995), and sustained depletion of satellite cells that can give rise to replicative progeny in tissue culture (Heslop *et al.* 2000). This thesis has demonstrated that the myogenic activity of single myofibre grafts originates from satellite cells which lose their myogenic potential after exposure to 18 Gy irradiation, thereby further implicating radiation-sensitive satellite cells as the phenotype whose absence prevents generation of functional MPC in irradiated muscles.

6.4 Evidence for MPC outside the satellite cell compartment

The numbers of desmin⁺ cells obtained from enzymatically-disaggregated muscles exceeded the number of satellite cells likely to have been present in each muscle (Chapter 3). This suggests that uninjured adult muscles contain MPC both within and outside of the satellite cell niche. *In vitro*, single myofibres were found to generate a greater proportion of satellite cell-like reserve progeny than satellite cells exist *in vivo*. Speculatively, satellite cells may generate both new satellite cells and MPC outside the niche. That non-satellite cell MPC are probably present in uninjured adult muscles suggests that they are not a transitory population which exists only during regenerative events, but form a permanent compartment.

The myogenic satellite cells in single myofibre grafts were found to be sensitive to radiation (Chapter 3). The results of a previous study showed that muscle also contains a radiation-resistant population of myogenic cells (Heslop *et al.* 2000). In Chapter 4, proliferative cells cultured from irradiated but otherwise undamaged adult muscles were found to be desmin⁺, but did not express donor markers, and were therefore likely to have been derived from the host tissue. This supports the view that radiation-resistant MPC are committed and resident within the tissue, rather than derived from the myogenic recruitment of endogenous or exogenous non-muscle phenotypes in response to acute damage. Radiation-resistant MPC can give rise to satellite cells (Heslop *et al.* 2000), but this thesis has demonstrated that grafts of radiation-sensitive satellite cells also efficiently generate new satellite cells. Speculatively, trafficking may occur between satellite cells and radiation-resistant cells such that the cells of what is essentially a single population acquire different

functional attributes according to their residency within two alternative niches. Whilst the ability of radiation resistant MPC to give rise to satellite cells has been established, the potential of radiation-sensitive satellite cells to conversely generate radiation-resistant MPC is not known. The implied existence of two closely-related, yet separable, subsets is analogous with the stem cell system of the adult skin epidermis, whereby two functionally similar subpopulations of stem cells exhibit behavioural and phenotypic heterogeneity that is linked to differences in anatomical location (Blanpain *et al.* 2004).

6.5 A proposed model for the role of satellite cells in adult muscle regeneration

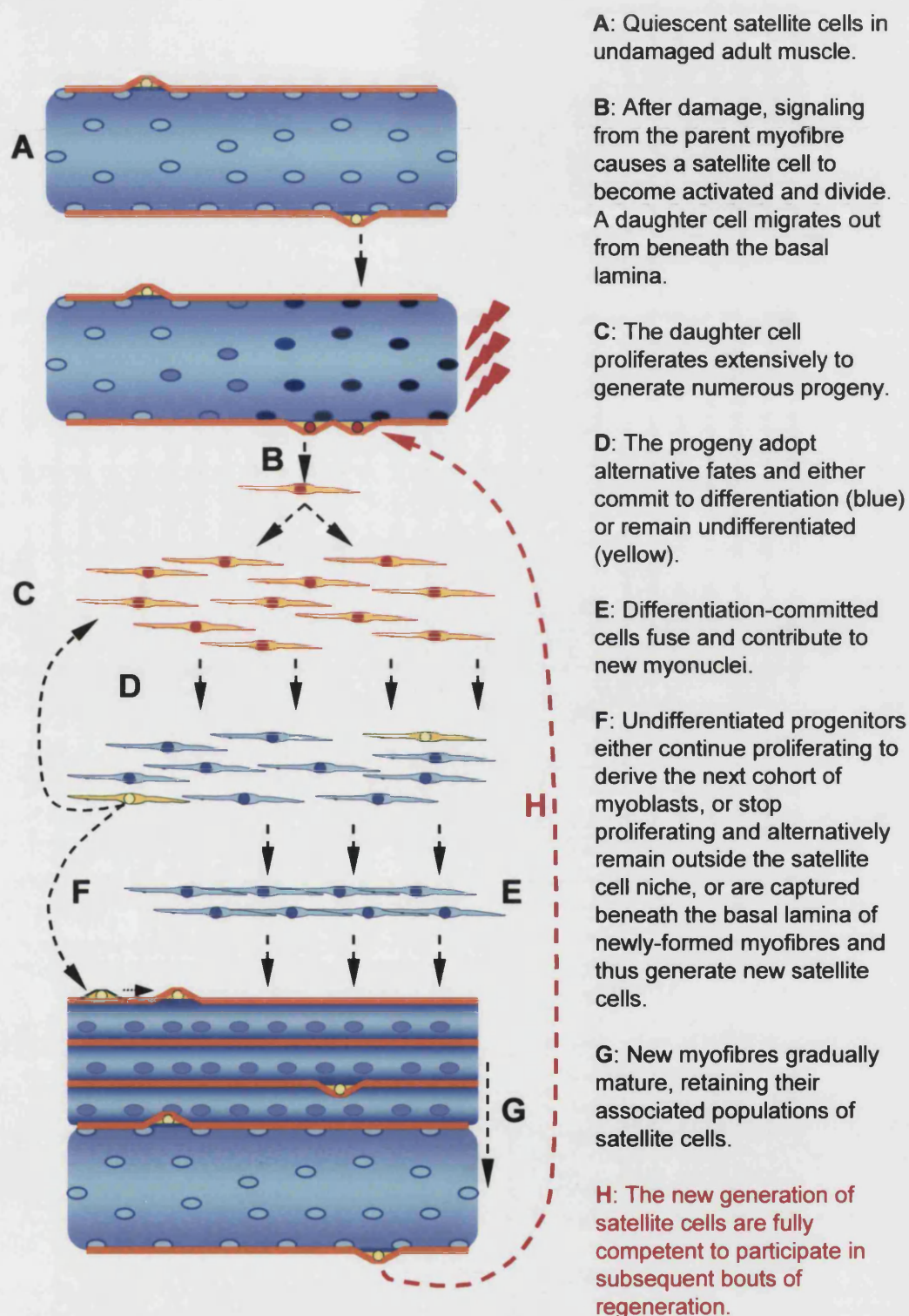


Fig. 6.1. A proposed model for the mechanism by which satellite cells constitute a self-sufficient source of myonuclei in adult muscles.

6.6 Satellite cell heterogeneity between different muscles

In Chapter 3, whilst the amounts of muscle generated from grafts of primary isolated myoblasts did not differ according to whether the cells were prepared from pooled EDL, soleus or TA muscles, muscle formation from grafted single myofibres exhibited striking inter-muscle heterogeneity. At the three week timepoint, soleus myofibres (with 22 satellite cells) formed muscle in 75% of graft sites, whereas EDL and TA myofibres, (with 7 and 10 satellite cells respectively), formed muscle in 32% and 33% of graft sites. These differences appear to correlate directly with differences the numbers of satellite cells typically present in each graft.

The large differences between the amounts of muscle formed by myofibres of different origin were unexpected, and cannot be explained fully by differences in numbers of satellite cells. At the three week timepoint, whereas a proportion of EDL and soleus myofibre grafts contributed to more than 100 new myofibres, no TA myofibre graft contributed to more than 11 new myofibres. This difference was found to be extremely significant. However, in experiments described in Chapter 5, where muscles were harvested at the five week timepoint, some muscles engrafted with TA myofibres contained more than 150 donor-derived myofibres. At this later timepoint, there was no statistically-significant difference between the total numbers of dystrophin⁺ myofibres formed by grafts derived from different muscles. However, when the numbers of newly-regenerated (dystrophin⁺ neonatal myosin⁺) myofibres were compared, there were significantly more newly-regenerated myofibres in TA myofibre-engrafted muscles than in EDL or soleus myofibre-engrafted muscles. Together, the data described in this thesis suggest that firstly, muscle formation is spontaneously augmented over time, and secondly, that the progeny of TA satellite cells chiefly differ from the progeny of EDL and soleus satellite cells in terms of their rate of proliferation (or possibly rate of differentiation), rather than in terms of absolute myogenic potential. One week after notexin treatment, TA single myofibre grafts generated smaller numbers of new myofibres than EDL or soleus single myofibre grafts, a difference which might also have resulted from a slower proliferative rate.

In vitro, the progeny of EDL and soleus satellite cells proliferate at similar rates, though the larger numbers of satellite cells associated with individual soleus myofibres accordingly generate larger absolute numbers of progeny (Zammit *et al.* 2002). There is no corresponding data available for TA satellite cells. However, since exposure to serum mitogens synchronously activates 98% of satellite cells within 24 h of myofibre isolation (Zammit *et al.* 2002), the tissue culture environment may not be the ideal means of modelling the *in vivo* phenomenon where inter- or intra-graft asynchrony of activation could be an important factor.

Other workers have reported that the efficiency of muscle formation is increased where donor myoblasts and host muscle are matched for fibre type compatibility (Peterson & Huard 2000, Qu & Huard 2000). The data described in Chapter 3 directly contradict this finding, in that TA satellite cells grafted into TA muscles formed muscle with initially lower efficiency than EDL or soleus satellite cells grafted into TA muscles. In addition, though it has previously been reported that a subset of soleus satellite cells preferentially differentiate into slow myofibres (Rosenblatt *et al.* 1996), this thesis has shown that after grafting into irradiated TA muscles, soleus satellite cells do not generate slow myofibres *de novo* but predominantly generate fast fibre-types, many of which moreover express the 3F-*nLacZ*-2E transgene. This finding suggests that any endogenous commitment is overridden by signals from the host tissue.

The different murine skeletal muscles are of oligoclonal embryonic origin (Eloy-Trinquet & Nicolas 2002, reviewed, Buckingham *et al.* 2003). It has been demonstrated that transplanted myoblasts can migrate between different muscles (Watt *et al.* 1987, Jockusch & Voigt 2003) and that satellite cells can be derived from bone marrow grafts (LaBarge & Blau 2002, Dreyfus *et al.* 2004). This thesis has shown that adult satellite cells have distinct muscle-specific qualities which are retained after transplantation into a different muscle. Whilst the TA and EDL muscles are anatomically adjacent (see **Fig. 2.1**), their satellite cell populations appear to be discrete. This finding suggests that firstly, functional satellite cells are not frequently derived from outside the tissue, and secondly, that inter-muscle trafficking of satellite cells is also infrequent.

Pax3⁺ Pax7⁺ cells are thought to represent a separate lineage to Pax3⁻ Pax7⁺ myogenic cells in adult skeletal muscle (personal communication, D. Montarras). In Chapter 4, it has been shown that Pax3-GFP⁺ cells can give rise to satellite cells which retain robust expression of Pax3-GFP protein. Since Pax3-GFP⁺ cells are frequent in the donor diaphragm muscle but infrequent in the host TA muscle, this is further evidence that the MPC populations of different adult muscles have distinct qualities which are retained independently of their external environment, and speculatively could reflect different embryonic origins.

6.7 Evidence for functionally-distinct subsets of satellite cells

In some of the experiments described in Chapter 5, 100% of single myofibre grafts were found to give rise to donor-derived products after regeneration of engrafted muscles was stimulated by notexin damage. This suggests that technical failure was not a major contributory factor in other experiments where only a proportion of grafts generated muscle. Local environmental differences in the myopathic host graft site (Tanabe *et al.* 1986, Coulton *et al.* 1988, reviewed, Partridge 1997) are likely to have been a partial determinant of myogenic recruitment, but there is no obvious reason why this factor should generate the evident variability between grafts derived from different muscles.

In contrast to soleus and TA myofibres, grafts of EDL myofibres were never found to generate donor-derived products in 100% of engrafted muscles. The highest frequency occurred after notexin treatment, and was 75%. Only EDL myofibre-engrafted muscles were examined at the extended twelve week timepoint; even under these conditions the frequency was only 56%. All EDL myofibres (n=77 examined) were found to have at least one Pax7⁺ associated satellite cell (Chapter 3). The lack of muscle formation by some EDL myofibres therefore implies that not all satellite cells form muscle after grafting. Since EDL myofibres have 7 associated satellite cells, soleus myofibres have 22 satellite cells and TA myofibres have 10 satellite cells, the maximum observed frequencies of muscle formation (100% for soleus and TA myofibre grafts, 75% for EDL myofibre grafts) could be accounted for by a hypothetical subset which forms only 10% of the compartment, and is therefore absent from some EDL myofibres whilst being present on the vast majority of soleus

and TA myofibres. This subset could either differ with regards to its ability to survive grafting, or differ in terms of its innate myogenic potential.

Functional heterogeneity of satellite cells has previously been identified by other workers (Rantanen *et al.* 1995, Schultz 1996, Rouger *et al.* 2004). Rantanen *et al.* (1995) showed that though proliferation does not commence until about 24 h after injury, some myoblasts upregulate expression of MyoD and myogenin within 8 h. This was taken as evidence for two separate subsets of satellite cells: one population which rapidly commits to differentiation and has only limited subsequent proliferative ability, and another more stem cell-like population that has the ability to undergo more extensive proliferation (Rantanen *et al.* 1995). Rouger *et al.* (2004) carried out clonal analysis of myoblasts derived from turkey muscles. It was found that about 8% of clones formed a highly-proliferative subset which preferentially fused into mature myofibres (Rouger *et al.* 2004). The proliferative subset described in these two studies (Rantanen *et al.* 1995, Rouger *et al.* 2004) could be analogous to the highly-proliferative satellite cells described in this thesis.

Schultz (1996) administered BrdU to juvenile rats. He found that whilst the majority population of satellite cells incorporated BrdU within a five day period, about 20% formed a slow-cycling subset which were labelled more slowly, and were postulated as the source of renewal of the majority population (Schultz 1996). Similarly, Rouger *et al.* (2004) found that about 50% of turkey myoblast clones cycled more slowly than the rest of the population, and had lower fusion indices. Slow-cycling clones were thought to generate satellite cells more frequently than other clones (though the lack of phenotypic markers in this study renders the observation slightly equivocal) (Rouger *et al.* 2004). The single myofibre grafts employed in this thesis each contained multiple satellite cells. Therefore hypothetically, satellite cells and muscle could have been generated separately by different clones. Nevertheless, the ability to participate in two or more regenerative events is evidence that the graft-derived satellite cells or MPC had latent myogenic potential.

6.8 Therapeutic implications

The data presented in thesis predict that in the mouse, satellite cells are the main source of new myonuclei in regenerating adult muscles. The extent to which these findings extrapolate to human skeletal muscle could be assessed using xenografts of human single myofibres (Bonavaud *et al.* 2002) into highly-immunodeficient host mouse strains such as the RAG2^(-/-)/γ-chain^(-/-) (Cooper *et al.* 2001) which are tolerant of human cells.

Historically, the apparent efficacy of MTT in mouse models (Partridge *et al.* 1989, Morgan *et al.* 1990) was not recapitulated by human clinical trials (Gussoni *et al.* 1992, Karpati *et al.* 1993, Tremblay *et al.* 1993, Mendell *et al.* 1995, Miller *et al.* 1997). It was found that though viable cells derived from the graft had persisted in patient muscles several months after transplantation, they had apparently failed to differentiate (Gussoni *et al.* 1997). In mouse models, myoblasts form muscle with greater efficiency in irradiated than in non-irradiated graft sites (Morgan *et al.* 1990, Kinoshita *et al.* 1994, Gross *et al.* 1999), and this thesis has demonstrated that single myofibres harbour a phenotype which is completely non-myogenic in non-irradiated graft sites. Irradiation was not used in human trials; perhaps the presence of the patients' own satellite cells inhibited the donor cells from homing to the niche required for them to initiate an active myogenic program.

Single myofibre grafts could represent a refinement over grafts of cultured myoblasts in future clinical trials of MTT. However, there are a number of practical problems which would need to be overcome. Whereas human myoblasts can be isolated from needle biopsies, isolation of viable single myofibres would probably require a more invasive surgical procedure. In addition, it would not be feasible to treat patient muscles with 18 Gy irradiation, though speculatively, the minimum dose required might be lower than this.

6.9 Future work

The single myofibre graft technique is a model system for investigating the behaviour of satellite cells during muscle regeneration. The most precise way of investigating the potential of stem cells is to use single cell transplantation (Osawa *et al.* 1996, Matsuzaki *et al.* 2004). The myofibres of the murine FDB muscle typically have only one satellite cell (personal communication, P. Gailly), and could therefore be used to perform grafts of single defined satellite cells. Single satellite cell transplantation would be a simple and robust method of clonal analysis, and would avoid the problems associated with viral labelling strategies described in Chapter 4.

A very robust method of demonstrating self-renewal is to carry out serial transplantations, whereby donor cells are re-isolated from a first host, and their continued potential examined in a second host. This might be achieved using a donor mouse strain in which satellite cells are marked by GFP expression, and would therefore be identifiable in living myofibres isolated from the first host. More refined experiments such as these could be used to analyse satellite cell behaviour with more precision and in greater detail.

The single myofibre graft technique could be used to investigate the contribution of satellite cells to other lineages, either using a donor strain with a ubiquitously-expressed genetic marker, or strains with genetic markers of specific lineages. Likewise, the technique could be employed to investigate the relationship of satellite cells with other putative stem cells within muscle tissue, such as muscle SP (Asakura *et al.* 2002) and the radiation-resistant population (Heslop *et al.* 2000).

6.10 Concluding remarks

This thesis has demonstrated that anatomically-defined satellite cells exhibit several stem cell-like qualities, including the ability to generate large numbers of differentiation-competent progeny, the potential to self-renew their own compartment, and the capacity to respond to damage by undergoing rapid clonal expansion and efficiently regenerating new muscle tissue. These findings show that the satellite cell niche is an important determinant of stem cell potential, and predict that satellite cells are the basal origin of new myonuclei in regenerating adult muscles.

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Appendix

Notes on statistical techniques

Statistical analyses were carried out using the computer program INSTAT.

Mann-Whitney test

The parametric t-test is correctly used to compare pairs of treatment populations that have a normal distribution and similar variability, but does not reliably compare populations that are not normally distributed or have unequal SDs. Since the data sets described in this thesis are not normally distributed and in many cases have dissimilar SDs, the Mann-Whitney test was used as a non-parametric (distribution-free) alternative to the t-test. This test requires that the sample populations have similar, but not necessarily normal, distributions i.e. they may be either positively or negatively skewed, but to a similar degree in each population. It can be used to compare two populations of different sizes. Mann-Whitney tests whether there is a difference between two population medians. The total data points from the two populations are ranked together in order of size. If two data points are tied, the average rank is given to each. The sum of ranks for each population forms the test statistic (W). The p value is adjusted to account for tied values. A p value of >0.05 indicates that the two populations are significantly different from each other (Watt 1997).

Kruskal-Wallis test

Kruskal-Wallis is a non-parametric ANOVA test that is used to compare multiple populations. It differs from standard parametric ANOVA in that whilst it is a less sensitive test, it can be used to compare populations that are not normally distributed, but are either positively or negatively skewed. However, the different populations are required to have similarly-shaped distributions. The total data points derived from all the treatment populations are ranked together, and the mean rank of each treatment population calculated. The test compares the mean rank of each treatment population with the mean rank of the total population, generating a 'z value' for each treatment

population. A p value of <0.05 indicates that at least one population is significantly different from the other populations (Fisher & van Belle 1993, Watt 1997).

Where p was <0.05 , Dunn's multiple comparisons test was used to resolve the origin of the significant difference. Dunn's test compares individual pairs of treatments within the data set and generates a p value for each pair. Where $p \leq 0.05$, the two paired populations are significantly different from one another.

Kolmogorov-Smirnov two sample test

The Kolmogorov-Smirnov non-parametric test is used to compare the distributions of two populations one or both of which does not follow the normal distribution. Data within each population are ranked as empirical cumulative distributions such that the distributions of two unequally sized populations can be compared. The value of the Kolmogorov-Smirnov test statistic (*KS*) is derived from the maximum absolute difference between the two empirical cumulative distribution functions. The test statistic is not efficient where the t-test applies. Where $p \leq 0.05$, the distributions of the two populations are significantly different from one another. The test is most reliable in the absence of tied data points (Fisher & van Belle 1993).

Addenda

References

Page 41: Cell-cycle regulation of Myf5 expression in C2 cells was originally reported in Lindon C., Montarras D., & Pinset C. (1998) Cell cycle-regulated expression of the muscle determination factor Myf5 in proliferating myoblasts. *J. Cell Biol.* 140, 111-118.

Pages 48 and 150: Sajko *et al.* (2004) refers to the following omitted publication: Sajko S., Kubinova L., Cvetko E., Kreft M., Wernig A. & Erzen I. (2004) Frequency of M-cadherin-stained satellite cells declines in human muscles during aging. *J. Histochem. Cytochem.* 52, 179-185.

Definitions

“Stem cell”: adult stem cells were originally defined as cells capable of maintaining a tissue by generating differentiation-competent progeny and undergoing repeated self-renewal throughout the normal lifespan of an organism (see page 30). So far only HSC have been demonstrated to fulfill these criteria robustly. However, both HSC and several other adult cell types are capable of generating several sorts of differentiated cell, and many authors now regard such multipotency as a defining characteristic of stem cells when combined with self-renewal. By these criteria not only HSC but certain primitive epidermal and neural cells can be regarded as adult stem cells, though in these cases it is not yet known whether self-renewal is maintained throughout an entire lifespan.

“Satellite cell niche”: here refers to the anatomical situation between the basal lamina and sarcolemma of myofibres by which satellite cells were originally defined (Mauro 1961).

“Phenotype”: is strictly defined as the observable characteristics of an organism resulting from the interaction between genotype and environment. In this thesis the term is used more loosely to define cell populations by observable characteristics such as the expression of specific marker proteins.

“Lineage”: a lineage is defined as the descendants of one individual. Cells which differ with regards to Pax3 expression (4.5) appear to form separable adult populations, but each population could have derived from multiple progenitors. Therefore a description of Pax3⁺ and Pax3⁻ muscle cells as separate lineages is inaccurate.

Note on possible inter-muscle heterogeneity of satellite cells

In Chapter 3, grafts of EDL and soleus single myofibres were occasionally found to give rise to very large clusters of muscle whereas grafts of TA single myofibres never gave rise to large clusters. However, the difference between the amounts of muscle formed by EDL and TA single myofibres was not found to be statistically significant using the Kruskal-Wallis and Dunn's tests. Consequently it is possible that the apparent inter-muscle heterogeneity resulted from chance alone. In Chapter 5, when analysed at a later timepoint TA myofibre grafts generated significantly larger amounts of newly-regenerated muscle than EDL myofibre grafts, though the total amounts of muscle formed did not differ. Therefore though the findings described in this thesis provide some evidence of satellite cell inter-muscle heterogeneity, further experiments, possibly combined with improved methods of data analysis, are required before such heterogeneity can be demonstrated unequivocally.